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DEVELOPMENT OF A VERSATILE SILICON-BASED BIOSENSOR PLATFORM FOR PATHOGEN DETECTION

presented by

FINNY P. MATHEW

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Biosystems Engineering

E C Sloccilja

Major Professor's Signature

May 3, 2006

Date

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DEVELOPMENT OF A VERSATILE SILICON-BASED BIOSENSOR PLATFORM FOR PATHOGEN DETECTION

By

Finny P. Mathew

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
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Department of Biosystems and Agricultural Engineering

2006

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ABSTRACT

DEVELOPMENT OF A VERSATILE SILICON-BASED BIOSENSOR PLATFORM FOR PATHOGEN DETECTION

By

Finny P. Mathew

Biosensors are portable diagnostic tools used for the rapid detection of pathogens that may require up to 2-7 days to detect otherwise. Biosensors are important in the fields of human and animal diagnostics, bioterrorism preparedness, food and water safety, as well as environmental safety. So a versatile silicon-based biosensor platform for the detection of bacteria that could be used with different biological detecting elements and transducers was fabricated and demonstrated. Silicon (0.01 ohm-cm, p-type) was etched in an electrochemical cell containing hydrofluoric acid solution using anodizing conditions of 5 mA/cm^2 for 1 h to fabricate nano-tubular Si (NTS). NTS chips were functionalized into biosensors using biological sensing elements such as enzymes, antibodies and nucleic acids. A chemiluminescence-based enzyme assay was adapted to the biosensor system for the detection of *E. coli* resulting in a lower detection limit of 10^2 CFU within 30 min. The NTS-based platform was also functionalized into an optical immunosensor to successfully detect a pure culture of *Salmonella* Typhimurium within 10 min. Finally, a DNA-based NTS biosensor was developed using an electrochemical transducer for the detection of *Salmonella* Enteritidis. The NTS DNA biosensor was able to detect *S.* Enteritidis DNA with a lower limit of detection of 1 pg/μl for PCR-amplified DNA and 10 pg/μl for pure culture-extracted DNA. The electrochemical detection process was completed within 60 min. The versatility of the NTS platform for pathogen

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detection was successfully demonstrated using three different biological sensing elements (enzyme, antibody, and DNA) and two detection methods (optical and electrochemical). Development of commercial biosensing devices using the NTS platform will strengthen biosecurity measures, enable improvement in human and animal health as well as quality assurance in food industry, while also reducing costs associated with loss of productivity, food product recalls and lawsuits.

Dedicat

Dedicated to my dear wife and my family in India, Chicago, and East Lansing

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ACKNOWLEDGMENTS

First of all, I thank my Lord and Savior, Jesus Christ, for the opportunity to pursue this degree, and for the strength and wisdom given to complete it. I would also like to thank everyone who helped me through this process. I want to extend my heartfelt thanks to my family - my parents, brother and sister as well as their families for their love and support. The same measure of thanks goes to my spiritual family here in East Lansing and Chicago without whose help it would have been tough getting through those low times. Also, celebration of all the joyous moments during the tenure of my degree would not have been possible without you all. I want to thank all of you who prayed for me during the course of my PhD program, couldn't have done it without you.

I would also like to thank Dr. Evangelyn C. Alocilja for being a mentor in more ways than one. Without your personal life example, constant encouragement and advice, this endeavor would definitely not have succeeded.

I would like to thank my labmates - Lisa, Maria, Stephen, and Zarini who helped me in the lab, and also were friends I could talk to while spending the many hours in the lab. I also should thank Lori Kindschy for her help in providing journal articles in the electronic format; you helped me save a lot of time.

Lastly, I have to acknowledge the love of my life - Sojo, my wife. You came into my life during the final stage of my PhD program and enriched my days with your phone calls, smile and jokes. You have also spurred me on to complete the corrections of this document for final submission.

Thank you very much, everyone!!

TABLE OF CONTENTS

LIST OF TABLES

Table 5-11. In
co
vo

Table 5-12. In
co
vo

Table 5-13. In
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Table 5-14. In
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LIST OF FIGURES

Figure C-41.

Figure C-42.

Figure C-43.

Figure C-44.

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Si – Si

LIST OF ABBREVIATIONS

CFU – colony forming units

h – hour/hours

min – minute/minutes

NTS – nano-tubular silicon

s – second/seconds

Si – Silicon

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CHAPTER 1: INTRODUCTION

The causes of foodborne illness include viruses, bacteria, parasites, fungi, toxins, and metals with the symptoms ranging from mild gastroenteritis to life-threatening neurological, hepatic, and renal problems. According to a report from the Centers for Disease Control and Prevention (CDC), it is estimated that foodborne diseases cause approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths in the United States each year. Researchers at the Economic Research Service (ERS) of the United States Department of Agriculture (USDA) estimate that the total annual medical cost associated with foodborne illness caused by pathogens (known and unknown) is $6.5-$9.4 billion.

The diagnosis of a foodborne disease is usually confirmed by cultures of stool or blood, which takes 2-3 days. Foodborne disease outbreaks lead to huge economic losses due to costs associated with lost work hours, lawsuits, and product recalls. Thus, detection of any foodborne pathogen is necessary before the contaminated product reaches the market.

Traditional methods are laborious, require technical expertise, and often several biochemical and serological tests have to be performed to identify pathogens, making these methods very time consuming (2-7 days). Rapid methods being developed usually take about 18-24 h (including the time required for sample processing and pre-enrichment).

Biosensors are portable diagnostic tools used for the rapid detection of metabolites, drugs, hormones, antibodies, and antigens. Biosensors are defined as analytical devices incorporating a biological material intimately associated with or integrated within a

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physicochemical transducer or transducing microsystem. While many of the biosensor applications are in the medical field, recent developments have been in the detection of pathogens especially for bioterrorism prepraredness.

Various platforms have been employed to fabricate biosensors, such as silicon (Si), silicon dioxide, fiber optics, filter paper, quartz, and glass. Nano-tubular Si (NTS) has recently been investigated as a potential platform for biosensor applications. Most of the recent developments in porous silicon sensors carry out a deposition step to fill the pores with metallic, dielectric or semiconducting oxide, enzyme, or molecular receptor films. This process has resulted in porous silicon sensors capable of detecting or measuring penicillin, alkali metal ions, humidity, and hydrocarbons. However, the use of NTS as a biosensor platform for detecting pathogens has not been reported.

This study was focused on developing a NTS-based biosensor platform and demonstrating its versatility by using three different biological sensing elements aimed at three different target pathogens and two transduction principles. The biosensor was fabricated using microfabrication and electrochemistry techniques and had the following characteristics: high sensitivity and specificity with an assay time of 60 min or less. The fabrication of NTS, its functionalization into biosensors using different biological detectors, and biosensor testing with *Escherichia coli* and two *Salmonella enterica* strains using optical and electrochemical transducers are described here.

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CHAPTER 2: LITERATURE REVIEW

2.1 MICROBIOLOGICAL DETECTION METHODS

2.1.1 Introduction

More than 200 diseases are known to be transmitted through food, and these are caused by 40 different foodborne pathogens, including fungi, viruses, and bacteria (Mead *et al.*, 1999). The Centers for Disease Control and Prevention (CDC) estimates that foodborne diseases cause approximately 76 million illnesses, including 325,000 hospitalizations and 5000 deaths in the US each year (Mead *et al.*, 1999). The estimated annual cost of human illness caused by a group of seven pathogens (including six bacteria: *Salmonella*, *Listeria*, *Campylobacter*, *E. coli* O157:H7, *Staphylococcus aureus*, and *Clostridium perfringens*) commonly associated with foodborne outbreaks is $5.6-9.4 billion annually (Buzby *et al.*, 1999).

Traditional methods for the detection of bacteria involve the following basic steps: preenrichment, selective isolation, and biochemical screening, as well as serological confirmation for certain pathogens. Hence, a complex series of tests is often required before any identification can be confirmed. These methods are laborious and may require a certain level of expertise to perform. The results of such tests are often difficult to interpret and not available on the time scale desired in the food quality assurance or clinical laboratory. Food sample testing also presents problems with the inadequacy of sampling protocols, and the presence of organic matter, as well as non-target bacteria. Therefore, the detection of target foodborne pathogens (for example, *Salmonella*) may be problematic due to low numbers present and may result in a false culture negative response. Moreover, four to seven days are generally required to confirm the presence of

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the pathogens (Food and Drug Administration, 2000), which has safety, cost, and quality implications for the food, medical, and biodefense sectors. Rapid detection methods for pathogens have hence become a necessity.

2.1.2 Rapid Detection Methods

New rapid detection technologies can be very sensitive, but analysis time is usually lengthy. For example, the polymerase chain reaction (PCR), a molecular method, can be used to amplify small quantities of genetic material to determine the presence of bacteria. The PCR method is extremely sensitive and can be used for various target pathogens, but requires pure samples and hours of processing and expertise in molecular biology (Meng *et al.*, 1996; Sperveslage *et al.*, 1996; Tsukamoto and Kanki, 1999; Almeida and Almeida, 2000; Narimatsu *et al.*, 2001; Hong *et al.*, 2003; Tims and Lim, 2003).

In response to this problem, considerable effort is now directed towards the development of methods that can rapidly detect low concentrations of pathogens in water, food and clinical samples. For this purpose, several instruments have been developed using various principles of detection, e.g., chromatography, infrared or fluorescence spectroscopy, bioluminescence, flow cytometry, impedimetry, and many others (Nelson, 1985; Wyatt, 1995; Hobson *et al.*, 1996; Perez *et al.*, 1998). Initial instruments developed for bacterial identification counted the cells by microscope or by flow cytometry; infrared spectroscopy; measuring physical parameters by piezocrystals, impedimetry, redox reactions, optical methods, calorimetry, ultrasound techniques and detecting cellular compounds such as ATP (by bioluminescence), DNA, protein and lipid derivatives (by biochemical methods), radioactive isotopes (by radiometry) (Nelson, 1985; Rossi and Warner, 1985; Ramsay and Turner, 1988; Rodrigues and Kroll, 1990; Ding *et al.*, 1993; Sharpe, 1994; Swaminathan and Feng, 1994; Hobson *et al.*, 1996; Wang *et al.*, 1997;

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Fratamico *et al.*, 1998; Gunasekera *et al.*, 2000). Among these, the primary physico-chemical methods of bacterial identification are those that involve the detection of some naturally occurring component of the bacterium. For example, the Microbial Identification System (MIS; Microbial ID, Inc., Newark, DE) uses gas chromatography to produce a fatty acid profile for detection and identification of microorganisms (Swaminathan and Feng, 1994; Crist Jr. *et al.*, 1996).

The enzyme-linked immunosorbent assay (ELISA) is probably the most commonly used immunological assay because of its versatility, sensitivity (ability to detect small amounts of antigen or antibody), specificity (ability to discriminate between closely related but antigenically different molecules), and ease of automation. Chemicals and substrates used in ELISA are safer than radioisotopes used in radioimmunoassay. The process can be easily automated for performance of large numbers of tests. The VITEK immunodiagnostic assay system (VIDAS®, bioMérieux, Inc., Durham, NC) is an automated, qualitative, enzyme-linked fluorescent immunoassay method. Comparative studies with a variety of food types have found favorable correlation between conventional test methods and the VIDAS system (Blackburn *et al.*, 1994; Curiale *et al.*, 1997; De Medici *et al.*, 1998; Walker *et al.*, 2001). Wyatt *et al.* (1996) observed a 70-80% agreement between the ELISA and the culturing method. The ELISA had a false-positive rate of 17% and a false-negative rate of 26%, which appeared to be due to insufficient growth of *Salmonella* spp. in the preenrichment broth employed in the ELISA, rather than lack of recognition by the antibodies.

Real-time PCR approaches have recently emerged as a preferred DNA-based diagnostic tool in both medical and agricultural fields (Marin *et al.*, 2004; Skof *et al.*,

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2004; Wada *et al.*, 2004; Whiley *et al.*, 2004; Connor *et al.*, 2005; Farrell *et al.*, 2005; Penders *et al.*, 2005; Rudi *et al.*, 2005; Uchida *et al.*, 2005). Real-time quantitative PCR is increasingly being used for pathogen detection and quantification in foods (Norton, 2002). There are still two major challenges with the widespread use of PCR for quantitative diagnostics. The detection limit is mainly determined by the amount of material that can be amplified in a single reaction. The other major limitation is the detection of DNA from dead cells (Herman, 1997; McKillip *et al.*, 1999; Nogva *et al.*, 2003). This is a particular problem for processed foods or foods subjected to long-time storage, wherein 'false' positives from dead cells could lead to unnecessary economic losses. Moreover, all the above methods require expensive, multiple, specialized, or large equipment.

2.1.3 Electronic Nose

'Electronic nose' systems have advanced rapidly during recent years, with most applications being within the food and beverage industries (Schaller *et al.*, 1998; Benedetti *et al.*, 2004; Garcia-Gonzalez *et al.*, 2004; Lui *et al.*, 2005; Marti *et al.*, 2005). Electronic nose systems are comprised of sophisticated hardware, with sensors, electronics, pumps, flow controllers, and software, such as data pre-processing, and statistical analysis (Schaller *et al.*, 1998). The sensor array of an electronic nose responds to both odorous and odourless volatile compounds. In the electronic nose, a computer collects the signal pattern from a sensor array, comprised usually of individual sensing elements with limited specificity, wherein the first pre-treatment of the data is carried out. These data are then further processed by suitable software based on artificial neural networks approach for training and learning (Benedetti *et al.*, 2004; Dutta *et al.*, 2004). At present, different detection principles (heat generation, conductivity, electrochemical,

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optical, dielectric, and magnetic properties) are used in the basic sensing elements of the electronic nose. The ideal sensors to be integrated in an electronic nose should fulfill the following criteria: high sensitivity (down to 10^{-12} g/ml), must respond to different compounds present in the headspace of the sample, high stability and reproducibility, short recovery time, easy calibration, and must be robust and portable (Schaller *et al.*, 1998). Artificial electronic noses have been used to assess cheese, and alcoholic beverage quality (Marti *et al.*, 2005; Trihaas and Nielsen, 2005), beef spoilage (Balasubramanian *et al.*, 2004), freeze damage in oranges (Tan *et al.*, 2005), and fruit ripeness (Brezmes *et al.*, 2005). Gibson *et al.* (1997) reported on the use of an array of 16 conducting-polymer resistive gas sensors to detect 12 different bacteria from cultures grown on agar plates. More recently, an electronic nose based on gas sensors coupled with an artificial neural network was developed for *E. coli* O157:H7 detection and its differentiation from non-O157:H7 isolates. The differences between the 'gas signatures' of the *E. coli* O157:H7 and the non-O15:H7 isolates were successfully detected using neural network algorithms (Younts *et al.*, 2002; Younts *et al.*, 2003).

Artificial/electronic noses offer advantages such as simplicity of design, long shelf life of sensors, capability for long-term continuous monitoring (for example, in produce storage quality studies), and non-destructive testing. However, they cannot be used for rapid detection of pathogens as they take a minimum of 12-24 h to detect significant changes in gas patterns emitted from a sample. Also, identifying a unique gas signature for each target pathogen may not be possible, and variations in gas emission within isolates of the pathogen increase the probability of false positive/negative outputs.

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2.1.4 Biosensors

The disadvantages with culture techniques, molecular and ELISA assays, as well as electronic noses have led to the development of several alternative methods (including biosensors) for the rapid detection of the presence of pathogens. Biosensors have emerged as highly promising for rapid diagnosis. The general function of a biosensor is to convert biological events into a quantifiable electrical response (Cahn, 1993). A biosensor consists of a platform, a biological sensing element, and a transducer. Transducers used in biosensors to report these biological events are electrical, electrochemical, optical, piezoelectric crystal, or acoustic wave in nature. The sensing element used in the construction of biosensors can vary from entire sections of tissue to individual molecules such as cells, enzymes, antibodies, and DNA oligonucleotides. Transducers used in biosensors with different kinds of biological sensing elements are discussed below.

2.1.4.1 Biological sensing elements

2.1.4.1.1 Whole cells and tissues

The use of tissue and whole (living) cells can impart certain advantages to a sensor, especially when subjected to suboptimal conditions. Biosensors based on whole cells as biorecognition elements are used as genotoxicity and toxicity sensors, targeting environmental pollutant analysis but have much far applications for food contaminant detection. Bacteria or yeast cells, genetically engineered to bear the *lux* or *luc* gene operon, express luminescence proteins such as the green fluorescence protein (GFP), and are used for detection of environmental pollutants, monitoring water quality, and stress response (Kohler *et al.*, 2000; Gu and Choi, 2001; Weitz *et al.*, 2001; Bechor *et al.*, 2002; Horsburgh *et al.*, 2002; Kostrzynska *et al.*, 2002). Other signal reporter systems such as

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bacterial luciferase, insect luciferase, β-galactosidase, and alkaline phosphatase have also been employed (Baeumner, 2003). Biran *et al.* (2000) developed an electrochemical biosensor for cadmium using genetically engineered microorganisms for non-luminescence signal generation with the *lacZ* gene. Plants and tissues as well as higher whole organisms have been used in some biosensor applications in recent years. These range from algae to soybeans, and nematodes. For example, Shvetsova *et al.* (2002) suggested the use of soybeans and their electrophysiological response to acid rain and other environmental stress conditions as a potential biosensor system. The biggest drawback of cell and tissue based biosensors is the problem of selectivity and slow response times, thus restricting their applicability.

2.1.4.1.2 *Enzymes*

The limitations of whole cell and tissue-based biosensors can be overcome by the use of enzymes, which represent the most commonly used sensing agent for chemical targets. Pesticides, especially insecticides, have traditionally been an application area for enzyme-based sensor systems. Many of them reach detection limits set forth by governmental agencies for environmental samples, infant food, etc., making them attractive alternatives to conventional analysis systems (Karube and Nomura, 2000; Rekha *et al.*, 2000). For example, acetylcholine esterases were used for the detection of organophosphates and carbamates in single-use devices such as screen-printed electrodes in both, environmental and food samples (Albareda-Sirvent *et al.*, 2001b, 2001a; Avramescu *et al.*, 2002; Schulze *et al.*, 2002a; Schulze *et al.*, 2002b; Marques *et al.*, 2004; Timur and Telefoncu, 2004). In food analysis, the freshness-test applied to the analysis of fish, sauerkraut, tomato paste, and instant food (Frebort *et al.*, 2000; Niculescu *et al.*, 2000; Kriz *et al.*,

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2002; Lange and Wittmann, 2002; Mitsubayashi *et al.*, 2004), antibiotic detection in milk (Gustavsson *et al.*, 2002), and detection of phytotoxins found in seafood (Botre and Mazzei, 2000) are some of the other enzyme-based biosensor applications reported.

Literature describing the detection of pathogenic bacteria using enzyme-based biosensors is limited. Sensors are found for the detection of *E. coli* O157:H7 and *Salmonella* Typhimurium with a bienzyme electrode chicken carcass wash water, ground beef, and fresh-cut broccoli (Yang *et al.*, 2001; Ruan *et al.*, 2002a). *Escherichia coli* was detected in environmental samples based on culture-enhanced amperometric and chemiluminescence principles with detection limits of about 10,000 cells per 100 ml of environmental sample within one working day (Nistor *et al.*, 2002). Low sensitivity and poor specificity of enzyme-based biosensors for pathogen detection limit the application of such techniques.

2.1.4.1.3 Antibodies and receptors

Biological sensing elements based on antibody and receptor molecules are grouped in this section, since their application area and functionality are very similar. Recently, the analytes targeted ranged from antibiotics in milk to general environmental monitoring to heavy metals to pathogens. The traditional application area of immunosensors is for pesticide detection in environmental samples and food matrices. Strachan *et al.* (2001) described the use of antibodies for the detection of atrazine, mecoprop, diuron, and paraquat emphasizing their superiority over other bioanalytical systems due to their functionality in aqueous and also organic solvents. Other environmental pollutants including PCBs were detected using screen-printed electrodes (Laschi and Mascini, 2002). In food contaminant analysis, sulfonamides (Bjurling *et al.*, 2000) and toxins, such as aflatoxin, fumonisin B1, cholera toxin, and staphylococcal enterotoxin B, were

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targeted in a variety of foods (Ho and Durst, 2000; Rasooly, 2001; Maragos, 2002; Ho and Durst, 2003; Medina, 2003; Rucker *et al.*, 2005). Antibody-based biosensors have been used for the detection of antibiotic and other drug residues in food samples, e.g., penicillin, streptomycin, and gentamicin in milk samples (Delwiche *et al.*, 2000; Haasnoot and Verheijen, 2001; Haasnoot *et al.*, 2002).

Finally, immunosensors have also been developed for pathogen analysis, but only some of these exhibit excellent detection limits with relatively short analysis times. A quartz crystal microbalance (QCM)-based biosensor was used to detect *Salmonella* sp. in milk samples with detection limits around 10^6 CFU/ml (Park *et al.*, 2000). *Listeria* and *Salmonella enterica* were detected with a similar detection limit by a surface plasmon resonance (SPR) biosensor (Koubova *et al.*, 2001b). QCM and SPR biosensors for different bacterial targets, such as *Pseudomonas aeruginosa*, *Bacillus cereus*, and *E. coli* O157:H7, were later developed by researchers (Vaughan *et al.*, 2003; Kim *et al.*, 2004; Su and Li, 2004; Su and Li, 2005), and showed no significant improvement in detection limits. In a dipstick-type assay, Park and Durst (2000) were able to detect *E. coli* O157:H7 in food matrices at a low detection limit of about 10^3 CFU/ml without any enrichment required.

An impedance biosensor chip for detection of *E. coli* O157:H7 was developed based on the surface immobilization of affinity-purified antibodies onto indium tin oxide (ITO) electrode chips, with a detection limit of 6×10^3 cells/ml (Ruan *et al.*, 2002b). Shah *et al.* (2003) developed an amperometric immunosensor with a graphite-coated nylon membrane serving as a support for antibody immobilization and as a working electrode. This approach was used for detection of *E. coli* with a low detection limit of 40 CFU/ml.

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An antibody-based conductimetric biosensor developed by Muhammad-Tahir and Alocilja also showed a similar detection limit of 50 CFU/ml for bacteria and 10^3 cell culture infective dose (CCID)/ml for virus within 10 min (Muhammad-Tahir and Alocilja, 2003a; Muhammad-Tahir and Alocilja, 2003b; Muhammad-Tahir *et al.*, 2005a, 2005b).

An antibody-based fiber-optic biosensor, to detect low levels of *L. monocytogenes* cells following an enrichment step, was developed using a cyanine 5-labeled antibody to generate a specific fluorescent signal. The sensitivity threshold was about $4.3x10^3$ CFU/ml for a pure culture of *L. monocytogenes* grown at 37°C. Results could only be obtained after 2.5 h of sample processing. In less than 24 h, this method could detect *L. monocytogenes* in hot dog or bologna naturally contaminated or artificially inoculated with 10 to 10^3 CFU/g after enrichment in buffered Listeria enrichment broth (Geng *et al.*, 2004). In another study, a microcapillary flow injection liposome immunoanalysis system (mFILIA) was developed for the detection of heat-killed *E. coli* O157:H7. Liposomes tagged with anti-*E. coli* O157:H7 and an encapsulating fluorescent dye were used to generate fluorescence signals measured by a fluorometer. The mFILIA system successfully detected as few as 360 cells/ml with a total assay time of 45 min (Ho *et al.*, 2004).

The ability to detect small amounts of materials, especially bacterial organisms, was demonstrated using micro electromechanical systems (MEMS) for the qualitative detection of specific *Salmonella enterica* strains with a functionalized silicon nitride microcantilever. Detection was achieved due to a change in the surface stress on the cantilever surface in situ upon binding of a small number of bacteria with less than 25

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adsorbed bacteria required for detection (Weeks *et al.*, 2003). A MEMS fabricated high-density microelectrode array biosensor, developed for the detection of *E. coli* O157:H7 using a change in impedance caused by the bacteria measured over a frequency range of 100 Hz-10 MHz, was able to detect and discriminate 10^4-10^7 CFU/ml (Radke and Alocilja, 2005).

The potential use of immunosensors is due to their general applicability, specificity and selectivity of the antigen-antibody reaction, and the high sensitivity of the method, depending on the detection method used. The antigen-antibody complex may be utilized in all types of sensors. The physichochemical change induced by antigen-antibody binding does not generate an electrochemically detectable signal. Therefore, enzymes, fluorescent compounds, electrochemically active substrates, or avidin-biotin complexes are used to label either the antigen or the antibody to detect the biological recognition event. Immunosensors cannot be employed to specifically detect viable cells. The antibodies used are selective to the epitope on the antigen. If the epitope is present on a living or dead microorganism, the antibody will capture the antigen and register a positive signal. In the case of bacterial foodborne pathogens that must be ingested by the host to cause disease, a positive result from non-viable cells may raise false alarm.

2.1.4.1.4 DNA and RNA

A nucleic acid (gene) probe is a segment of nucleic acid that specifically recognizes, and binds to, a nucleic acid target. The recognition is dependent upon the formation of stable hydrogen bonds between the two nucleic acid strands. This contrasts with interactions of antibody-antigen complex formation where hydrophobic, ionic, and hydrogen bonds play a role. The bonding between nucleic acids takes place at regular (nucleotide) intervals along the length of the nucleic acid duplex, whereas antibody-

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protein bonds occur only at a few specific sites (epitopes). The frequency of bonding is reflected in the higher association constant for a nucleic acid duplex in comparison with an antibody-protein complex, and thus indicates that highly specific and sensitive detection systems can be developed using nucleic acid probes (McGown *et al.*, 1995; Skuridin *et al.*, 1996). The specificity of nucleic acid probes relies on the ability of different nucleotides to form bonds only with an appropriate counterpart. Since the nucleic acid recognition layers are very stable, an important advantage of nucleic acid ligands as immobilized sensors is that they can easily be denatured to reverse binding and then regenerated simply by controlling buffer-ion concentrations (Graham *et al.*, 1992). Most nucleic acid biosensors are based on this highly specific hybridization of complementary strands of DNA and also RNA molecules.

The detection of bacteria and other pathogens in food, drinking water, and air, based on their nucleic acid sequences, has been explored using various detection systems. Biosensors have been developed to detect DNA hybridization at sub-picomolar to micromolar levels using gravimetric detection systems (Sung Hoon *et al.*, 2001). A quartz crystal nanobalance system could detect DNA hybridization at 0.3 nanogram levels using frequency shift nanogravimetric measurement (Nicolini *et al.*, 1997). Tombelli *et al.* (2000) developed a DNA piezoelectric biosensor for the detection of bacterial toxicity based on the detection of PCR amplified *aer* gene of *Aeromonas hydrophila*. The biosensor was applied to vegetables, environmental water, and human specimens. The biosensor was able to successfully distinguish between samples containing the pathogen and those not contaminated. Zhao *et al.* (2001) developed a QCM biosensor using 50 nm gold nanoparticles as the amplification probe for DNA

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detection in the order of 10 fM of target, which was higher than what has been ever reported using the same method. The high sensitivity was explained by the weight of the larger particles, and the larger area occupied by the larger particles that needed less target DNA for their binding. Another QCM biosensor applied to the detection of *E. coli* in water in combination with PCR amplification (of the *lac* gene) was able to detect a 10 fg of genomic *E. coli* DNA (few viable *E. coli* cells in 100 ml of water) (Mo *et al.*, 2002). When used for detection of *Hepatitis B virus*, Zhou *et al.* (2002) observed that the QCM could detect frequency shifts of DNA hybridization as a linear relationship in the range 0.02-0.14 μg/ml with a detection limit of 0.1 μg/ml, similar to the QCM biosensor developed by He and Liu (2004) for *Pseudomonas aeruginosa*. While QCM biosensors have been demonstrated to successfully detect bacterial and viral targets, they rely on PCR amplification of the target due to poor sensitivity, in addition to requiring 3-12 h for DNA hybridization and detection.

Optical biosensor systems developed for DNA detection exhibit higher sensitivity compared to the QCM biosensors. An automated optical biosensor system based on fluorescence excitation and detection in the evanescent field of a quartz fiber was used to detect 16-mer oligonucleotides in DNA hybridization assays. The detection limit for the hybridization with a complementary fluorescein-labeled oligonucleotide was 2×10^{-13} M (Abel *et al.*, 1996). Another optical fiber evanescent wave DNA biosensor used a molecular beacon (MB) DNA probe that became fluorescent upon hybridization with target DNA. The detection limit of the evanescent wave biosensor with synthesized complementary DNA was 1.1 nM. Testing with environmental samples was not performed (Liu and Tan, 1999). Liu *et al.* later developed MB-DNA biosensors with

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micrometer to submicrometer sizes for DNA/RNA analysis. The MB-DNA biosensor was highly selective with single base-pair mismatch identification capability, and could detect 0.3 nM and 10 nM of rat gamma-actin mRNA with a 105-μm biosensor and a submicrometer (0.1 μm) biosensor, respectively (Liu *et al.*, 2000).

Optical biosensors targeting RNA as the analyte offer an added advantage over traditional DNA-based detection methods, i.e., viable cell detection. Baeumner *et al.* (2003) detected as few as 40 *E. coli* cells/ml in samples using a simple optical dipstick-type biosensor coupled to Nucleic Acid Sequence Based Amplification (NASBA), emphasizing the fact that only viable cells were detected, and no false positive signals were obtained from dead cells present in a sample. The detection of viable cells is important in respect to safety, and also food and environmental sample sterilization assessments. Similarly, a biosensor for the protozoan parasite *Cryptosporidium parvum* was developed (Esch *et al.*, 2001). Hartley and Baeumner (2003) developed a simple membrane strip-based biosensor for the detection of viable *Bacillus anthracis* spores. The study combined the optical detection process with a spore germination procedure as well as a nucleic acid amplification reaction to identify as little as one viable *B. anthracis* spore in 12 h. A quantitative universal biosensor was developed on the basis of oligonucleotide sandwich hybridization for the rapid (30 min total assay time) and highly sensitive (1 nM) detection of specific nucleic acid sequences (Baeumner *et al.*, 2004). The biosensor consisted of a universal (polyethersulfone) membrane, a universal dye-entrapping liposomal nanovesicle, and two oligonucleotides – a reporter and a capture probe that could hybridize specifically with the target nucleic acid sequence. Limits of detection of 1 nM per assay and dynamic ranges between 1-750 nM were obtained. While

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the RNA-based biosensor can be an excellent tool for detection of viable bacterial cells, inherent disadvantages to the technique include the short life span of the mRNA target, high susceptibility to contaminants and inhibitors from environmental and food samples, and need for complex detection systems.

Other biosensors targeting DNA that have been developed include MEMS-based amperometric (Gau *et al.*, 2001) and high throughput PCR biosensors (Nagai *et al.*, 2001), carbon nanotube-based field effect transistor biosensor (Maehashi *et al.*, 2004), microcantilever-based cyclic voltammetry biosensor (Zhang and Li, 2005), pulsed amperometry- (Ramanaviciene and Ramanavicius, 2004), capacitance- (Berney *et al.*, 2000; Lee *et al.*, 2002), and absorbance-based biosensors (Mir and Katakis, 2005). Several electrochemical DNA biosensors have been developed that will be discussed further (Yan *et al.*, 2001; Cai *et al.*, 2002; Meric *et al.*, 2002; Ye *et al.*, 2003; Kara *et al.*, 2004; Lee *et al.*, 2004; Del Pozo *et al.*, 2005; Wong and Gooding, 2005).

2.1.4.2 Transducers

2.1.4.2.1 Bioluminescence sensors

This phenomenon of bioluminescence is the ability of certain enzymes to emit photons as a byproduct of their reactions. Bioluminescence may be used to detect the presence and physiological condition of cells. The potential applications of bioluminescence for bacterial detection involve the development of luciferase reporter phages that infect host bacteria, conferring a bioluminescent phenotype to previously non-bioluminescent bacteria. Bioluminescence systems have been used for detection of a wide range of microorganisms and environmental toxicity markers (Prosser, 1994; Prosser *et al.*, 1996; Ramanathan *et al.*, 1998; Weitz *et al.*, 2001; Bechor *et al.*, 2002; Bolton *et al.*, 2002; Shao *et al.*, 2002; Farre and Barcelo, 2003). Folley-Thomas *et al.*

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(1995) used the TM4 bacteriophage to detect *Mycobacterium avium* and *M. paratuberculosis*. However, a concentration of 10^4 cells was required to produce a detectable luciferase signal, and the response declined after 2 h. Using the same approach, *Salmonella* spp. and *Listeria* were also detected (Turpin *et al.*, 1993; Chen and Griffiths, 1996). A sensitive and specific method was developed for the specific detection of *Salmonella* Newport and *E. coli* (Blasco *et al.*, 1998) using ATP bioluminescence. Increased sensitivity was obtained by focusing on the bacteria's adenylate kinase as the cell marker instead of ATP. Light emission was proportional to cell numbers over three orders of magnitude, and 10^3 cells were readily detectable in a 0.1 ml sample. The bioluminescence approach is an attractive approach due to its extremely high specificity and the inherent ability to distinguish viable from non-viable cells. However, the main disadvantages are the relatively long assay time and lack of sensitivity when low numbers of bacteria are to be detected.

2.1.4.2.2 Optical biosensors

Optical transducers are attractive for application to direct, label-free, detection of bacteria. These sensors are able to detect minute changes in the refractive index or thickness that occur when cells bind to receptors immobilized on the transducer surface. Several optical techniques have been reported for detection of bacterial pathogens including: waveguides (Lukosz *et al.*, 1991; Zhou *et al.*, 1991; Amato *et al.*, 2000; Rowe-Taitt *et al.*, 2000; Demarco and Lim, 2002; Lim, 2003), surface plasmon resonance (Kai *et al.*, 2000; Haes and Van Duyne, 2002; Chen *et al.*, 2003; Medina, 2003; Leonard *et al.*, 2004), ellipsometry (Swenson, 1993; Bae *et al.*, 2005), the resonant mirror (Watts *et al.*, 1994; Watts *et al.*, 1995; Hirmo *et al.*, 1998; Lathrop *et al.*, 2003), and the interferometer (Schneider *et al.*, 1997; Liu *et al.*, 2002).

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Swenson (1993) utilized an ellipsometric technique for the development of a label-free instrument for detection of bacteria. The main component of the biosensor was an optical unit that consisted of an excitation source and a photodiode detection system. Metabolizing bacteria would result in an increased CO_2 concentration, which in turn affected an emulsion of aqueous colorimetric pH indicator, thus modulating the fluorescence detected at the photodiode.

The resonant mirror is another technique that can be used for direct detection of bacteria (Cush *et al.*, 1993). It is based on the use of a thin layer (~100 nm) of a high refractive index dielectric material and a thicker layer (~1 mm) of low refractive index material. At certain angles of incidence, light may be coupled into the high refractive index layer where it undergoes multiple internal reflections at the top interface, allowing an element of light, the evanescent wave, to penetrate to the sample overlayer. On reflection, the light undergoes a phase change, and by monitoring the angle at which this occurs, it is possible to detect changes within the evanescent field. A resonant mirror immunosensor was used for detecting *S. aureus* at 10^7cells/ml within 5 min (Watts *et al.*, 1994). The use of the resonant mirror biosensor for direct and rapid detection of DNA-DNA hybridization was also demonstrated, with the lowest detectable concentration of target oligonucleotide (40-mer) being 9.2 nM (Watts *et al.*, 1995).

Schneider *et al.* (1997) described an evanescent wave interferometer that used a single planar wave of polarized light. Light from a diode laser source was coupled into the waveguiding film as a single broad beam. The light then passed through multiple sensing regions on the surface of the chip. An array of integrated optical elements was used to combine light passing through adjacent regions that were functionalized with

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specific or non-specific receptors. Using this technique it was possible to directly detect *Salmonella* Typhmurium in the range of $5x10^8$ CFU/ml with a detection time of 5 min. The main advantage of the above techniques is their short detection time, however this is compromised by their severe lack of sensitivity.

2.1.4.2.3 Piezoelectric biosensors

Piezoelectric (PZ) biosensor systems, in principle, may be used for direct label-free detection of bacteria (Babacan *et al.*, 2002; Zhou *et al.*, 2002; Lin and Tsai, 2003; He and Liu, 2004; Kim *et al.*, 2004). This technology offers a real-time output, simplicity of use, and cost effectiveness. The theoretical basis of piezoelectricity is based on coating the surface of the PZ sensor with a selectively binding substance, for example, antibodies to bacteria, and then placing it in a solution containing bacteria. The binding of bacteria to the antibodies will increase the mass of the crystal while proportionally decreasing the resonance frequency of oscillation.

PZ immunosensors were developed for *Vibrio cholerae (Carter et al., 1995)*, *Salmonella* Typhimurium (Babacan *et al.*, 2002), *L. monocytogenes (Jacobs et al., 1995)*. A technique using a QCM sensor coated with a thin culture medium film was also developed and applied to determine *Staphylococcus epidermidis* in the range of 10^2–10^7 cells/ml (Bao *et al.*, 1996). However, the PZ membrane was not strong enough to withstand several autoclavings. Prusak-Sochaczewski *et al.* (1990) developed a QCM biosensor for the detection of *S.* Typhimurium wherein the coated crystal was stable for six to seven assays. For repeated use, the bound bacteria were removed from the crystal by washing with 8 M urea. A flow-injection system, based on a PZ biosensor was also developed for detection of *S.* Typhimurium by immobilizing anti-*Salmonella* spp. antibody onto a gold-coated quartz crystal surface (Ye *et al.*, 1997). The biosensor had

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responses of 23–47 Hz in 25 min with a dynamic detection range of $5.3x10^5$ to $1.2x10^9$ CFU/ml.

Possible limitations of PZ technology include the lack of specificity, sensitivity, and interferences from the liquid media where the analysis takes place. Disadvantages of PZ sensors are the relatively long incubation time of the bacteria, the numerous washing and drying steps, and the problem of regeneration of the crystal surface. Regeneration of crystals may not be an issue if small crystals can be manufactured at low cost so that disposable transducers are economically feasible.

2.1.4.2.4 *Electrical impedance biosensors*

Microbial metabolism usually results in an increase in both conductance and capacitance, causing a decrease in impedance. Therefore, the concepts of impedance, conductance, capacitance, and resistance are different ways of monitoring the test system, and are all inter-related (Silley and Forsythe, 1996; Milner *et al.*, 1998). The relationship between impedance (Z), resistance (R), capacitance (C), and frequency (f) for a resistor and a capacitor in series is expressed as follows (Bataillard *et al.*, 1988): $Z^2 = R^2 + \frac{1}{(2\pi fC)^2}$. Impedance is usually measured by a bridge circuit, with a reference module that measures and excludes non-specific changes in the test module. The reference module serves as a control for temperature changes, evaporation, changes in amounts of dissolved gases, and degradation of culture medium during incubation. This method is well suited for detection of bacteria in clinical specimens, to monitor quality and detect specific food pathogens, also for industrial microbial process control, and for sanitation microbiology (Swaminathan and Feng, 1994; Silley and Forsythe, 1996). This technique has been used for detecting microbial metabolism (Dezenclos *et*

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al., 1994; Palmqvist *et al.*, 1994), and for detecting the concentration and physiological state of bacteria (Desilva *et al.*, 1995; Dupont *et al.*, 1996; Ehret *et al.*, 1997). Current instruments usually detect active metabolizing bacteria when 10^6–10^7 cell/ml are present in the culture media. Applied to bacterial detection in urine, concentrations of 10^5 cells/ml can be detected using the impedance technique with a detection time of 2.5 h.

A biosensor for real-time monitoring of concentration, growth, and physiological state of cells in culture media was proposed by Ehret *et al.* (1997). This biosensor was based on impedance measurement of adherently growing cells on interdigitated electrode structures. Cell density, growth, and long-term behavior of cells on the electrodes changed the impedance of the biosensor due to the insulating property of the cell membrane. The presence of intact cell membranes on the electrodes and their distance to the electrodes determined the current flow, and thus the sensor signal. The biosensor provided information about spreading, attachment, and morphology of cultured cells. A similar detection principle was used by Radke and Alocilja (2005) to develop a high-density microelectrode array biosensor for detection of *E. coli* O157:H7. One disadvantage of using the impedence method is most analyses takes 20–25 h to complete.

2.1.4.2.5 *Fluorescence labeled biosensors*

Microorganisms are immunogenic due to the presence of proteins and polysaccharides in their outer coats. This permits the development of immunoassay techniques for bacterial detection. In fluorescent immunoassays (FIA), fluorochrome molecules are used to label antibodies. The fluorochrome absorbs short-wavelength light and then emits light at a higher wavelength that can be detected using fluorescent microscopy. Fluorescein isothiocyanate and rhodamine isothiocyanate-bovin serum albumin are the most common fluorochromes used to tag antibodies. Direct and indirect

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detection methods are used to test bacteria-containing samples (Colwell *et al.*, 1985; Donnelly and Baigent, 1986; Brayton *et al.*, 1987; Kaspar and Tartera, 1990). Food samples tested by FIA are typically from enrichment cultures because of insufficient bacterial numbers in the original sample to be directly detected, and interference caused by food particulates that produces background fluorescence. Identification of bacteria by fluorescent immunoassays (FIA) takes advantage of the high degree of specificity inherent in the immunological reaction.

An immunomagnetic assay system (IMAS) was developed for detection of virulent bacteria in biological samples (Yu and Bruno, 1996; Vernozy-Rozand *et al.*, 1997). The IMAS consisted of a magnetic separator for capturing the antigen, and an electrochemiluminescent detector. The IMAS was coupled to a flow cytometer and a continuous fluorimeter. This approach, like other chemiluminescence techniques, offered high signal-to-background ratios, and was comparable in sensitivity to radioisotopic methods. It had an advantage over other chemiluminescence techniques of being initiated by a voltage potential, and thus providing better-controlled luminescence (Yu and Bruno, 1996). The total assay time was one hour. The sensitivity of the IMAS for *Bacillus anthracis* spores, *E. coli* O157:H7, and *S.* Typhimurium detection was approximately 100 cells/ml in phosphate buffered saline (PBS) and 1000 cells/ml in biological samples. Decreased sensitivity of the IMAS detection in biological samples was due to sample interference.

2.1.4.2.6 *Flow immunosensors*

Most microbial assays are currently based on solidphase enzyme-linked immunoassays (ELISA) using microtitration plates. This is a powerful analysis tool used in biomedical research due to its high reproducibility and possibility to simultaneously

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conduct a large number of assays (Hock, 1996). However, disadvantages of heterogeneous ELISA methods include the small sample volume (200 μl) that the microtitration plate holds and the long incubation time required for each ELISA step. Also, the sensitivity of ELISA methods is insufficient for direct measurement of bacteria and other microorganisms in the original samples. Because low numbers of pathogenic bacteria are often present in a biological sample, an analytical standard often used for pathogenic bacteria is to detect cells in 25 g of food (Wyatt, 1995; Food and Drug Administration, 2000). Therefore, in order to increase assay sensitivity, it would be desirable to concentrate the bacteria into a smaller volume prior to the assay or by growing a single cell into a colony. Several possible formats for concentrating cells in analytical systems were described by Wyatt (1995). The most attractive technique for the concentration of bacteria is membrane filtration in conjunction with flow systems (Duverlie *et al.*, 1992; Paffard *et al.*, 1996; Abdel-Hamid *et al.*, 1999a). This procedure, called the flow immunofiltration assay, can be an excellent alternative to traditional ELISA for detection of bacterial pathogens, because it not only overrides the effects of diffusional limitations, but also allows the concentration of bacteria on the membrane by filtering a large volume of the sample. Heterogeneous flow immunofiltration assays offer extremely accelerated binding kinetics (Morais *et al.*, 1997) due to high surface area to volume ratio, and the flowing stream actively bringing the sample in contact with the solid-phase antibody. Paffard *et al.* (1996) developed an apparatus for use in an enzyme linked immunofiltration assay (ELIFA) for the detection of *E. coli*. Quantitative bacterial detection was based on precipitated chromogen determined by scanning densitometry, and the ELIFA method was capable of detecting 10^3 bacterial cells within 40 min.

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Another flow injection immunosensor, developed for the determination of *E. coli* in artificially contaminated food samples (Bouvrette and Luong, 1995), was based on direct noncompetitive heterogeneous immunoassay of *E. coli* cells with an antibody column and a fluorescence detector. The advantage of this method was that bacterial concentrations could be determined without using any labeled compounds through the direct detection of the cell's β-D-glucuronidase activity. However, the detection limit for *E. coli* was on the order of $5x10^7$ CFU/ml, which is less than the detection limit of the standard ELISA procedure for microbial cells (typically 10^6 CFU/ml).

Another promising format of immunoassay is based on the use of flow injection systems and antibody coated magnetic particles. This technique can be easily automated, the analyses performed quickly and continuously, and the renewal of the sensing surface of immunosensor was easily accomplished. The system has been applied to detect *E. coli* O157 and *S.* Typhimurium (Brewster *et al.*, 1996; Gehring *et al.*, 1996; Yu and Bruno, 1996; Vernozy-Rozand *et al.*, 1997; Brewster and Mazenko, 1998; Perez *et al.*, 1998). This approach, though expensive, offers a number of advantages over the ELISA, including greater antibody binding capacity, higher sensitivity, reduction of assay time, and simpler operation.

2.1.4.2.7 *Genomic sensors*

The detection of specific DNA sequences provides the basis for detecting a wide variety of bacterial pathogens. Gene probes have been used in the detection of disease-causing microorganisms in water supplies, food, and in plant, animal or human tissues (Scheu *et al.*, 1998; Simpkins *et al.*, 2000; Eun *et al.*, 2002; Wang and Yeh, 2002; Jackson *et al.*, 2004; Kotlowski *et al.*, 2004). The original DNA hybridization test for

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bacteria in foods used a radioactively labeled probe (Feng, 1992). The main disadvatages of radiolabelled probes are the short-shelf life of P^{32}-labelled probes, high cost, hazards, and disposal problems associated with radioactive waste. The limitation of nucleic acid probes is also a problem associated with cultivating bacteria to a detectable level. Hybridization requires the presence of at least 10^5–10^6 bacteria in the sample to obtain a positive signal. Therefore, without pre-enrichment of the target organism, direct DNA hybridization approach does not provide the required sensitivity to detect bacteria at required levels (Tietjen and Fung, 1995). However, PCR extremely enhances the sensitivity of DNA probes, at least three orders of magnitude. To date, only methods involving PCR have been employed to detect foodborne pathogens. Bacteria can be detected directly, without cultivation, by extraction and isolation of nucleic acids from real samples, followed by hybridization with specific probes. Without any enrichment steps, the PCR method could detect less than 40 CFU/g of a given food sample (Tietjen and Fung, 1995). Alternative ways to detect nucleic acid hybridization by using non-radioactive labeled probes have been developed (Bruno and Kiel, 1999; Liu *et al.*, 2000; McKillip and Drake, 2000; Seo *et al.*, 2004).

Two aims of DNA-based biosensor development should be emphasized: a) an improvement over conventional nucleic acid assay performance, and b) the design of special gene probe techniques for special applications under special conditions. Based on the nature of the physical detection principle used in the transducer, genosensing systems can be classified as optical, gravimetric, and electrochemical. The principles of these transducer systems have been described above.

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Evanescent wave methods of total internal reflection fluorescence (TIRF) (Graham *et al.*, 1992) and LAPS (Lee *et al.*, 2000) were described as labeling methods for DNA assays. Direct (label-less) gravimetric monitoring of hybridization reactions has been demonstrated with SPR (Kai *et al.*, 2000; Wang *et al.*, 2004b) and piezoelectric acoustic wave devices (Zhou *et al.*, 2002; He and Liu, 2004). The Biacore system (Biacore International SA, Neuchâtel, Switzerland) is a commercially available optical sensor based on evanescent wave technology that has been used for the detection of DNA–DNA interactions (Kai *et al.*, 2000; Jongerius-Gortemaker *et al.*, 2002; Wang *et al.*, 2004b). The system uses SPR that arises in thin metal films under conditions of total internal reflection. In the sensing element of this instrument, a gold transducer surface is modified with a dextran matrix on which the biological probe is immobilized. Oligonucleotides are introduced within a fluid flow system. Hybridization is carried out at room temperature, and positive signals are obtained within several minutes (Jongerius-Gortemaker *et al.*, 2002).

Several electrochemical DNA biosensors have been developed (Cai *et al.*, 2002; Kerman *et al.*, 2002; Cai *et al.*, 2003a; Li and Hu, 2004; Wong and Gooding, 2005). Electrochemical detection of DNA hybridization usually involves monitoring of a current response resulting from the Watson–Crick base-pair recognition event, under controlled potential conditions. The probe-coated electrode is commonly immersed into a solution of a target DNA whose nucleotide sequence is to be tested. When the target DNA hybridizes, a duplex DNA is formed at the electrode surface. DNA hybridization is then detected via the increased current signal of an electroactive indicator (DNA intercalator) (Kerman *et al.*, 2002), the use of enzyme labels or redox labels (Napier *et al.*, 1997; Ju *et*

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al., 2003), or from other hybridization-induced changes in electrochemical parameters (for example, capacitance or conductivity) (Cai *et al.*, 2003b; Wei *et al.*, 2003).

Two types of hybridization are currently used: (i) pseudo-homogeneous hybridization, which can be achieved in systems with high surface-to-volume ratio, such as porous membranes and highly dispersed immobilization carriers; (ii) solid-phase hybridization, which is preceded by transfer to a membrane. The main disadvantage of solid-phase relative to pseudo-homogeneous hybridization is the longer time required and the need for several manipulations (Aubert *et al.*, 1997). The DNA hybridization method is expensive, complicated (multistep assay), time-consuming, and requires trained personnel to perform. There is also a problem of false amplifications from dead cells when testing samples subjected to sterilization/sanitization treatments.

Each biological sensing element and transducer has its advantages and limitations. The focus of the current research will be to develop a versatile biosensor platform using different biological sensing elements and transducer systems. This will demonstrate the potential for future development of a biosensor system which can employ multiple biological sensing elements and transducer systems to provide a self-validating device with built-in output redundancy. This would be helpful in applications where a low probability of false positives and negatives is a must, for example, for bioterrorism countermeasures. One of the key stages of the biosensor development will involve the immobilization of the biological sensing element.

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2.2 IMMOBILIZATION TECHNIQUES

The immobilization of the desired biological sensing element such as enzyme, antibody, and DNA probe is a key step in biosensor development. When the biological material produces the physicochemical changes in close proximity to a transducer, it can be successfully detected. Immobilization not only helps in achieving the required close proximity of the biomaterial with the transducer but also in stabilizing it for possible reuse. The biological material can be immobilized directly on the transducer or, in some cases, in membranes, which is subsequently mounted on the transducer. Biological materials can be immobilized either through adsorption, entrapment, covalent binding, cross-linking, or a combination of all these techniques (Hock, 1996; Cosnier, 1998; Thust *et al.*, 1999; Berney *et al.*, 2000; Cai *et al.*, 2004). Selection of a technique and platform depends on the nature of biological material, nature of substrate that produces the physicochemical change, and configuration of the transducer used.

2.2.1 Enzyme Immobilization

Enzymes have normally been immobilized either by covalent binding, through physical adsorption, or by cross-linking. Physical immobilization, a simple and easy method, is the adsorption of enzymes onto solid supports (for example, biosensor platform). The enzyme is dissolved in solution, and the solid material is in contact with the enzyme solution for a fixed period of time. The unadsorbed enzyme is then removed by washing with a buffer. The adsorption mechanisms are governed by electrostatic attraction, hydrophobic interaction, and van derWaal's forces (Choi, 2004). This method is simple, mild, and causes little or no enzyme inactivation; hence, the enzyme activity is maintained, and the sensitivity of the biosensor will be higher.

Various enzymes have been entrapped within cross-linked organic or inorganic polymers. The preparation is done by cross-linking of the polymer in the presence of the enzyme; thus, the enzyme can be physically entrapped in the micro-pores of the natural or synthetic polymer (Koronczi *et al.*, 2001; Ivekovic *et al.*, 2004). The size of the pores is small, and this can prevent larger enzyme from leaching but allows small analytes to pass through to react with the entrapped enzyme (Kuswandi *et al.*, 2001). When covalent binding or cross-linking is used for enzyme immobilization, the enzyme needs to be bound without significantly affecting its conformational flexibility, since minor alterations in conformational changes on binding a ligand to a protein molecule can often be used as a criteria in analytical determinations (Bonnington *et al.*, 1995). Glycoprotein enzymes such as glucose oxidase, peroxides, and invertase have been covalently bound via their carbohydrate moiety (Melo and Dsouza, 1992; Husain and Jafri, 1995) preventing the chemical modification of functional groups in the protein moiety of the enzyme. Entrapment and adsorption techniques are also useful when cells or cellular organelles are used (Doktycz *et al.*, 2003; Li *et al.*, 2004). Novel sol-gel synthetic techniques have been developed to immobilize enzymes in stable, optically transparent matrices (Lobnik and Cajlakovic, 2001). The enzyme is added to the sol-gel solution. After the sol-gel has been gelled, the enzyme will be entrapped within the polymeric network of the porous gel (Wolfbeis *et al.*, 2000). Enzyme-based sol-gel biosensors have been developed for detection of glucose (Zaitoun, 2005), hydrogen peroxide (Wang *et al.*, 2004a; Xu *et al.*, 2004), cholesterol (Shi *et al.*, 2005), and creatinine (Pandey and Mishra, 2004). Gelatin has also been used as a natural polymeric support (Deshpande *et al.*, 1986; Svitel *et al.*, 1998). However, the method often followed with gelatin is cross-linking

using glutaraldehyde (GA), which is known to inactivate some of the enzymes requiring amino groups for activity.

Chemical immobilization of enzymes can be achieved by covalently bonding the enzymes to functionalized solid materials or intermolecular cross-linking of the biomolecules. The binding of the enzymes to the solid support is achieved by activating the surface of the support, followed by coupling of the enzyme to the activated surface. The excess and unreacted enzymes are removed by washing with a buffer. Cyanuryl chloride (2,4,6-trichlorotriazine) is a versatile reagent that has been used for the activation of surfaces containing hydroxy groups and subsequent immobilization of enzyme layers. Similarly, solid surfaces can be functionalized to carboxylic acid or amino groups with subsequent attachment with enzymes. The enzyme is coupled with the amino residues surface by glutaraldehyde (Choi, 2004). In general, this technique provides permanent and stable attachment of enzyme molecules to the support, leading to longer shelf life and stable biosensors; however, the enzymatic activity often is not as good as in case of immobilized enzymes prepared by physical immobilization as some of the enzymes are prone to be deactivated in the coupling reaction (Kuswandi *et al.*, 2001).

2.2.2 Antibody Immobilization

Antibody immobilization is a vital step in successful development of an immunosensor. The immobilization method must preserve the biological activity of the antibody and enable efficient binding. These methods can be grouped into three main categories: (1) adsorption; (2) immobilization via entrapment in acrylamide membranes (Ye *et al.*, 1997; Vikholm, 2005); and (3) immobilization via glutaraldehyde (GA) and other cross-linking agents (Bhatia *et al.*, 1989; Narang *et al.*, 1997; Nashat *et al.*, 1998; Slavik *et al.*, 2002; Radke and Alocilja, 2005).

Attachment of antibodies on quartz or glass can be achieved by simple adsorption; however, the immobilized proteins suffer partial denaturation, and tend to leach or wash off the surface (Bhatia *et al.*, 1989; Huang *et al.*, 2004; Zhou and Muthuswamy, 2004). Also, this approach does not provide permanent attachment because the complex is weakly bound to the solid support by adsorption.

The selection of an effective method for a particular biosensor requires a detailed study of different immobilization methods. For example, when selecting a method for piezoelectric biosensors, the approach should address: (1) the best biological material for immobilization; (2) the optimum immobilization parameters for the highest yield and reproducibility of the attached layer on the crystal; and (3) the effect of the immobilization layers on the frequency and surface characteristics of the quartz crystal (Babacan *et al.*, 2000). The results of these individual studies can then be combined to select the best method for the particular application.

Two methods have been reported to have the best potential for use in piezoelectric flow injection analysis (FIA) system, immobilization on a precoated crystal with polyethylenimine (PEI) (Ye *et al.*, 1997; Lin and Tsai, 2003; Tsai and Lin, 2005), and immobilization through Protein A coupling (Boltovets *et al.*, 2002; Su and Li, 2005). Protein A is a cell wall protein, produced by most strains of *Staphylcoccus aureus*. Protein A is a directed immobilization method due to its natural affinity towards the Fc region of IgG molecules. This does not block the active sites of the antibodies for analyte binding (Babacan *et al.*, 2000). In the PEI–glutaraldehyde (GA) method, immobilization of antibodies is achieved via surface aldehyde groups of GA on a quartz crystal precoated

with PEI. Glutaraldehyde is a homobifunctional cross-linker. In this method, the antibodies are randomly oriented and bound to the active surface (Babacan *et al.*, 2000).

The self-assembled monolayer (SAM) technique offers one of the simplest ways to provide a reproducible, ultra thin, and well-ordered layer suitable for further modification with antibodies, which has potentials in improving detection sensitivity, speed, and reproducibility (Chen *et al.*, 2003; Su and Li, 2004; Vikholm, 2005).

Covalent binding is the preferred method of attaching an antibody to a surface due to the strong, stable linkage that is formed. Hydroxyl groups on the biosensor platform surface provide sites for covalent attachment of organic molecules. Several investigators have modified surface hydroxyl groups to provide a functionality that would react directly with antibodies (Bhatia *et al.*, 1989; Shriver-Lake *et al.*, 1997; Nashat *et al.*, 1998). Coating the biosensor surface with a silane film provides a method for modifying the reactive hydroxyl groups on the surface to attach cross-linking agents. Silanes reportedly used include 4-aminobutyldimethylmethoxysilane, 4-aminobutyltriethoxysilane, 3-mercaptopropyltrimethoxysilane, mercaptomethyldimethylethoxysilane, and 3-aminopropyltriethoxysilane with cross-linkers such as glutaraldehyde, N-γ-maleimidobutyryloxy succinimide ester, N-succinimidyl-3-(2-pyridyldithio) propionate, N-succinimidyl-(4-iodoacetyl)aminobenzoate, and succinimidyl 4-(p-aleimidophenyl) butyrate (Bhatia *et al.*, 1989; Shriver-Lake *et al.*, 1997). Silanes utilized for the modification of the biosensor surface (gold, silicon, glass) introduce amino groups on it, which in turn provide reaction sites for covalently bonding to glutaraldehyde. Antibodies are then immobilized through Schiff base to the cross-linker, glutaraldehyde. Glutaraldehyde has been known to form

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large polymers. These reactive polymers may bind many residues and form multi-protein complexes (Bhatia *et al.*, 1989). These effects are likely to interfere with protein function, lowering the antibody activity on the biosensor surface. To obviate the problem, Sportsman and Wilson (1980) coated glass with glycidoxypropylsilane and oxidized the silane to produce aldehyde groups reacting directly with antibody. This method needs fewer functionalization steps and less processing time, while providing a functionalized biosensor with a high activity of immobilized antibodies.

2.2.3 DNA Immobilization

Another immobilization technique used with DNA probes (as well as antibodies) is based on the avidin-biotin chemistry (Wang *et al.*, 2004b). Avidin is a basic glycoprotein with an isoelectric point (pI) of approximately 10, originally isolated from chicken egg white. It is also found in the tissues of birds. Biotin is a naturally occurring vitamin found in every living cell. The tissues with the highest amounts of biotin are the liver, kidney, and pancreas. Yeast and milk are also high in biotin content. Cancerous tumors have more biotin than normal tissue (Savage *et al.*, 1994). The most commonly used method in SPR-based optical sensing is the immobilization of biotinylated DNA probes or antibodies onto a layer of streptavidin. Streptavidin is a biotin-binding protein isolated from culture broth of *Streptomyces avidinii.* Streptavidin binds four moles of biotin per mole of protein, and has a pI of approximately 5-6 (Savage *et al.*, 1994). Streptavidin is covalently linked to carboxylated dextran fixed onto the gold surface of the SPR chip on a self-assembled monolayer of 11-mercaptoundecanol (Bianchi *et al.*, 1997; Jordan *et al.*, 1997; Silin and Plant, 1997; Tombelli *et al.*, 2000; Feriotto *et al.*, 2002; Gao *et al.*, 2004; Ruan *et al.*, 2004). This method is very efficient in terms of sensitivity, selectivity, and stability of the realized sensor chip for DNA hybridization detection (Silin and Plant,

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1997; Kukanskis *et al.*, 1999; Mariotti *et al.*, 2002), but remains a time consuming procedure (5 days) if one starts from the gold surfaces.

Many transduction principles use gold surfaces, for example, SPR, piezoelectric, and electrochemical sensing. Self-assembly of terminally thiol-labeled oligonucleotides onto gold surfaces offers a direct method for chemisorption of DNA probes onto transducer surfaces based on the formation of gold-thiolate bonds (Herne and Tarlov, 1997; Yang *et al.*, 1998; Steel *et al.*, 2000; Cho *et al.*, 2001; Hianik *et al.*, 2001; Satjapipat *et al.*, 2001). Wang *et al.* (2004b) investigated the suitability of direct coupling of probes by self-assembly of terminally thiol-labeled oligonucleotides for SPR-based sensor. The performance (sensitivity, reproducibility, and specificity) of thiol chemistry was compared to the streptavidin-biotin binding chemistry using commercially available SPR instruments, BIACORE X™, and SPREETA™. The results obtained on the BIACORE X™ device with synthetic oligonucleotides, using the thiolated probe method, showed higher reproducibility and stability, good sensitivity, specificity, and lifetime of the sensor chip (more than 100 measurements were performed on the same modified chip), when compared to the streptavidin-biotin method.

The research reported in this study will investigate the performance of different immobilization methods with enzyme, antibody, and DNA probes on the biosensor platform to be developed. These techniques include adsorption, cross-linker mediated immobilization using glutaraldehyde, and direct coupling of antibodies and DNA probe to the silanizing agent. Once the biological sensing element has been immobilized, the biosensor functionalization step is completed. The biosensor will then be used to detect the target using appropriate transduction methods.

2.3 CHEMILUMINESCENCE AND BIOSENSORS

2.3.1 Chemiluminescence

Optical immunosensors are one of the most popular sensors for bioanalysis. This is due to the advantages of applying visible radiation, the nondestructive operation mode, and the rapid signal generation and reading (Luppa *et al.*, 2001). Luminescence is the release of energy by matter in the form of light. Luminescence transducers combine high selectivity and sensitivity to monitor changes of various parameters in the analytical system, such as concentration of the analytes and products, concentration of quenchers, protolytic and complexation reactions, membrane potential, and hydrophobicity or hydrophilicity. Main approaches in luminescence sensing utilize either luminescence intensity or lifetime measurement (Lin and Ju, 2005). When energy in the form of light is released from matter because of a chemical reaction, the process is called chemiluminescence.

Chemiluminescence is the phenomenon observed when the vibronically excited product of an exoergic reaction relaxes to its ground state with emission of photons. The chemical reaction produces energy in sufficient amounts (approximately 300 kJ/mol for blue light emission and 150 kJ/mol for red light emission) to induce the transition of an electron from its ground state to an excited electronic state. This electronic transition is often accompanied by vibrational and rotational changes in the molecule. In organic molecules, transitions from a π bonding to a π^* anti-bonding orbital ($\pi \rightarrow \pi^*$) or from a non-bonding to an anti-bonding orbital ($n \rightarrow \pi^*$) are most frequently encountered. Return of the electron to the ground state with emission of a photon is thus called chemiluminescence. The excited molecule can also lose energy by undergoing chemical

reactions, by collision, internal conversion or inter-system crossing. These radiationless processes are undesirable from an analytical point of view as they compete with chemiluminescence (Dodeigne *et al.*, 2000).

In general, the chemiluminescence reaction can be represented as:

$$A + B \xrightarrow{Catalyst} C^*$$

$$C^* \rightarrow C + h\upsilon$$

Chemiluminescence occurs as a result of the oxidation of certain substances, usually with O_2 or H_2O_2, to produce light in the absence of any exciting illumination. The optical transducer is configured to pick up the emitted light and transmitted to an optical detector for recording (Lin and Ju, 2005).

Chemiluminescent methods have been applied in routine food analysis, as well as in clinical and biomedical research, due to such advantages as no radioactive wastes, the relatively simple instrumentation required, the very low detection limit, and wide dynamic range (Baeyens *et al.*, 1998; Kricka, 2003). It is versatile and flexible to many different approaches. In general, two major chemiluminescent analytical techniques are used in clinical analysis: (1) luminol, dioxetanes, or acridinium ester as label to be used in chemiluminescent immunoassay; or (2) chemiluminescent detection techniques using horseradish peroxidase (HRP) or alkaline phosphatase as label for enzyme immunoassay (Thorpe *et al.*, 1985). The first technique detects directly a light flash of the chemiluminescent reagent to determine the analytes. In the second method, the chemiluminescent substrates undergo a peroxidase- or phosphatase-catalyzed oxidation in the presence of a suitable oxidant, and the detection limit for enzyme label is typically in attomolar range by the chemiluminescent detection. Several chemiluminescent sensors

and their application have been reported (Ramanathan *et al.*, 1998; Ye *et al.*, 2002; Liu *et al.*, 2003; Jain *et al.*, 2004; Lin *et al.*, 2004a, 2004b). Most of these sensors are based on immobilized enzymes, in which chemiluminescent reagents react with hydrogen peroxide released from the enzymatic reactions to produce a chemiluminescent light signal.

2.3.2 Chemiluminescent Immunosensors

When immunoreagents are immobilized, the chemiluminescent sensors are called chemiluminescent immunosensors (Marquette and Blum, 2000). In this case, the chemiluminescent reagents or enzymes (catalyze) are labeled to the antibody (or antigen) molecules. After the immunological reaction, the chemiluminescent intensity of the label is detected by chemiluminescent method for the quantitative analysis of antibody or antigen. Ye *et al.* (2002) developed a biosensor, consisting of a chemiluminescence reaction cell, a fiber optics light guide, and a computer-linked luminometer, used in conjunction with immunomagnetic separation for rapid detection of *Escherichia coli* O157:H7. HRP in the sandwich antibody-antigen complexes catalyzed the reaction between luminol and H_2O_2 in the reaction cell. The chemiluminescence biosensor was selective to *E. coli* O157:H7 with a detection limit of 1.8×10^2 CFU/ml, and the assay was completed within 1.5 h.

Figure 2-1 shows the simplified reaction mechanism of luminol, the substrate typically used for chemiluminescent immunoassays. The key intermediate is an α-hydroxyperoxide obtained by oxidation of the heterocyclic ring. The decomposition pattern of this intermediate, leading to the excited state and the light emission, is unique and depends only on the pH of the system. In contrast, the first step is strongly dependent of the composition of the medium (Merenyi *et al.*, 1990). In protic solvents (water, water

solvent mixtures or lower alcohols), various oxygen derivatives (molecular oxygen, peroxides, superoxide anion) can oxidize luminol derivatives but catalysis either by enzymes or by mineral catalysts is required. Catalysts employed for immunoassays include enzymes such as microperoxidase, myeloperoxidase, horseradish peroxidase, catalase, and xanthine oxidase (Kozlov *et al.*, 1990; Radi *et al.*, 1990; Rongen *et al.*, 1994). The bacterial peroxidase from *Arthromyces ramosus*, characterized by a very high turn-over, was claimed to provide a hundred times increase in sensitivity (Akimoto *et al.*, 1990; Kim *et al.*, 1991).

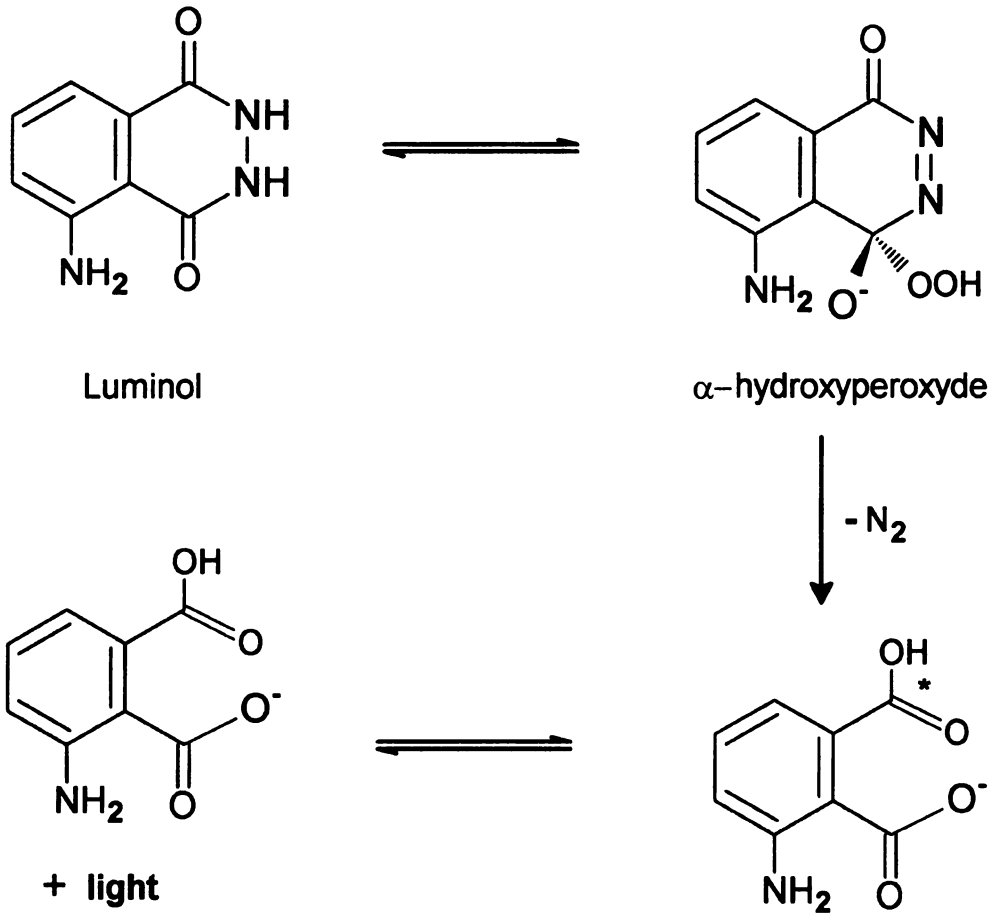

Figure 2-1. Simplified reaction mechanism of luminol: the key intermediate is an α-hydroxyperoxide obtained by oxidation of the heterocyclic ring. Its decomposition leads to the aminophthalate ion with light emission (Dodeigne *et al.*, 2000).

Many chemiluminescent immunoassay test kits and automated immunoassay analyzers have been developed and commercialized (Kricka, 2003). The fully automated, random-access, walk-way chemiluminescent immunoassay analyzers have been widely used in food and clinical analysis due to efficient use of labor, minimal sample and reagent manipulation, ease of automation, and flexibility (Rymer *et al.*, 1999; Hendriks *et al.*, 2000; Nandakumar *et al.*, 2000). Flow injection immunoassay (FIA) has been shown to be useful for improving cumbersome, time-consuming, and labor-intensive traditional immunoassay (Xu *et al.*, 1989; Pollema *et al.*, 1992; Gunaratna and Wilson, 1993). This technique has been applied to many fields such as food, pharmaceutical, environmental, and clinical assays (Bjarnason *et al.*, 1997; Abdel-Hamid *et al.*, 1999b; Burestedt *et al.*, 2000; Nandakumar *et al.*, 2000) due to the small volume required, the reduced sampling handling, good reproducibility, and easy automation for high sample throughput. In FIA heterogeneous immunoassays, a solid support is usually used to immobilize either the antibody or antigen. A wide variety of matrices, including inorganic surfaces, organic polymers, and other commercially available solid supports, have been used for the design of packing, while materials for preparation of flow injection immunosensors can be bead supports (silica, agarose, sepharose, and polystyrene particles with a magnetic nucleus) or membranes (Aberl *et al.*, 1992; Bouvrette and Luong, 1995; Abdel-Hamid *et al.*, 1999a; Choi *et al.*, 2000). These immunosensors have been used for detection of different targets such as *E. coli*, *E. coli* O157:H7, and *Salmonella*. The main disadvantages of this type of optical transducer are finite lifetime due to reagent consumption, and steady-state mass transfer and laminar flow characteristics required to get a constant signal.

The current research will focus on development of a chemiluminescent immuno-biosensor on a versatile silicon-based platform for detection of pathogenic bacteria such as *Salmonella*. The light output, recorded automatically using a luminometer connected to a computer, will be measured in a static (flow) environment to improve the sensor performance characteristics over existing flow injection immunoassay systems. Continuous automatic recording of the light output data will provide rapid results, expected within 5-10 min.

2.3.3 Chemiluminescent Enzyme-Based Sensors

In many instances, enzymatically generated chemiluminescence offers greater sensitivity and more rapid detection of viable cells than antibody-based immunoassays described above, as well as fluorometry, colorimetry, and radiolabeled probes (Van-Poucke and Nelis, 1995). The emission of light due to the chemiluminescence reaction between reagent and analyte has been employed in enzyme-based optical biosensors. The light emitted can be measured using charged couple device (CCD) cameras, photo-multiplier tube detectors, avalanche photodiodes, fiber optics, laser detectors, etc. After having compared different luminescent and non-luminescent detection methods, different authors have concluded that dioxetanes are among the most sensitive ones (Nelson and Kacian, 1990; Gillespie and Hudspeth, 1991; Kricka, 1991).

Figure 2-2 shows the decomposition mechanisms for 1,2-dioxetane compounds leading to generation of chemiluminescence. The dioxetanes decompose thermally, chemically or enzymatically into two carbonylic compounds, one of which is in the excited sate (Bronstein *et al.*, 1989a). Two distinct modes are observed (Figure 2-2). The diradical mechanism mainly occurs during thermal decomposition. Very high yields of excited states are obtained, but unfortunately, the often triplet excited state, T1, is rapidly

quenched in aqueous solutions, and therefore, of poor utility in diagnostic applications. Enzymatic or chemical decomposition is achieved through a chemically initiated electron exchange chemiluminescence (CIEEL) mechanism: a rigorous two-bond breaking concomitant process leads to an electronic redistribution and the formation of the two carbonylic products. Large singlet excited state (S1) versus T1 ratios are generally obtained, which makes this reaction much more efficient in aqueous solutions (Dodeigne *et al.*, 2000)

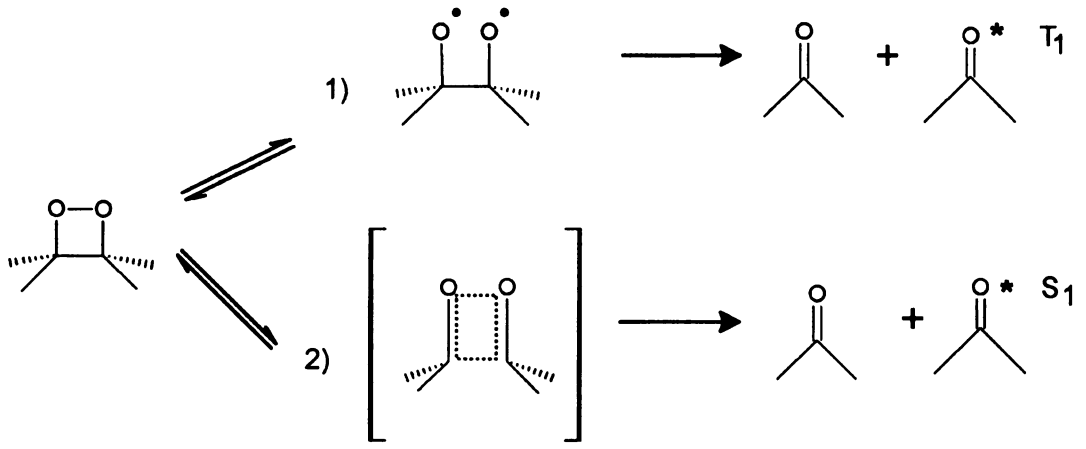

Figure 2-2. The two modes of decomposition of 1,2-dioxetanes: (I) the diradical mechanism and (II) the chemically initiated electron exchange chemiluminescence (CIEEL). The diradical mechanism most often generates triplet excited states (T1) while CIEEL generally results in singlet states (S1) (Dodeigne *et al.*, 2000).

For the reasons listed above, thermally unstable dioxetanes are not suitable for diagnostic applications. Chemically triggered (thermally stable) dioxetanes have attracted attention from end-users for enzyme-based assays. β-D-galactosidase and alkaline phosphatase enzymes, used for a long time as labels in immunoassays and DNA-based detection methods, were adapted to chemiluminescent detection using dioxetanes (Bronstein *et al.*, 1989b; Thorpe *et al.*, 1989). The most widely used dioxetanes, 3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane disodium salt

(AMPPD) and 3-(2'-spiroadamantane) - 4-methoxy - 4-(3"-β-D-galactopyranosyloxyphenyl) - 1,2-dioxetane (AMPGD), are the substrates for high turnover enzymes currently used in immunoassays, alkaline phosphatase and β-D-galactosidase, respectively (Bronstein *et al.*, 1989a).

In recent years, attention has shifted from the use of dioxetanes in immunoassays to enzyme-based assays for direct, easy, and highly sensitive detection of β-D-galactosidase. The *lacZ* gene from *Escherichia coli* is frequently used as a reporter gene in determining promoter strength in both transiently and stably transfected cells (Alam and Cook, 1990). Chemiluminescence-based methods are preferable to colorimetric and fluorometric assays for β-galactosidase detection as they are much more sensitive, and do not require sophisticated, expensive equipment. Jain and Magrath (1991) developed an AMPGD-based chemiluminescent assay for β-galactosidase that could detect as little as 2 fg of β-galactosidase activity in a single cell, stably transfected with a *lacZ*-expressing vector.

Dioxetanes have also been used for detection of *E. coli* by using the β-galactosidase enzyme (a metabolic product) as the marker. Van Poucke and Nellis (1995) developed a chemiluminometric assay of β-galactosidase in coliform bacteria, using AMPGD as the substrate. By this method, one coliform seeded in 100 ml of sterile water could be detected after nine hours of pre-enrichment followed by a 45-min enzyme assay in the presence of polymyxin B. Compared with fluorometry and colorimetry, chemiluminometry allowed the detection of a smaller number of cells, the increase in sensitivity being 4- and 1,000-fold, respectively (Van Poucke and Nelis, 1995).

Indirect chemiluminescence is also possible with dioxetanes. A very hydrophobic derivative of a fluorescer (5*N*-tetradecanoyl-aminofluoresceine) can be included in the micelles of the surfactant, hexadecyltrimethyl ammonium bromide (Lumi-Phos™ from Lumigen Inc., Southfield, MI), and used in the dioxetane formulation, or the fluorescer itself can be conjugated to the dioxetane ring (Beck and Koster, 1990). Lumi-Gal 530 (Lumigen, Inc.), a commercial formulation of 4-methoxy-4-(3-β-D-galactosidephenyl)spiro[1,2-dioxetane-3,2'-adamantane], reacts with β-galactosidase to generate indirect chemiluminescence (Figure 2-3). The stable dioxetane is enzymatically deprotected by β-galactosidase (in step 1) to produce an unstable form of dioxetane under alkaline conditions (in step 2). This unstable form decomposes in local hydrophobic environment producing the singlet-excited ester (in step 3). Indirect chemiluminescence results when the excited ester decays (step 4), and this energy is transferred to the fluorescer in the Lumi-Gal® 530 cocktail (Beale *et al.*, 1992).

Lumi-Gal 530 was shown to have a 20-fold greater sensitivity over other standard spectrophotometric assays when used with a pure β-galactosidase solution (Beale *et al.*, 1992). No further research has been reported for the development of a Lumi-Gal® 530-based chemiluminescent biosensor for *E. coli* detection using β-galactosidase as the marker, which will be the one of the aims of the research reported herein.

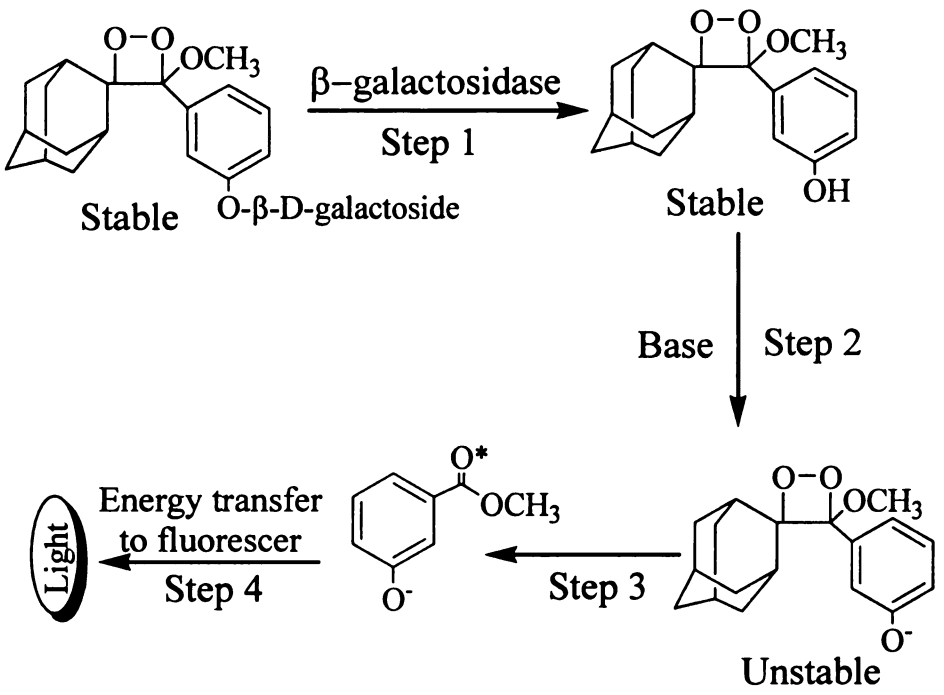

Figure 2-3. Reaction mechanism of Lumi-Gal® 530 in the chemiluminescent assay.

2.4 ELECTROCHEMICAL BIOSENSORS

Electrochemical biosensors are the most commonly used class of biosensors. Electrochemical transducers are based on a bio-interaction process, and during this process an electrochemical species, such as electrons, are consumed or generated producing an electrochemical signal, which can in turn be measured by an electrochemical detector. Electrochemical biosensors have been widely accepted in biosensing devices (Shah and Wilkins, 2003). Electrochemical sensors have some advantages over optical-based systems in that they can operate in turbid media, offer comparable instrumental sensitivity, and are more amenable to miniaturization. Modern electroanalytical techniques have very low detection limits (typically 10 nM) that can be achieved using small volumes (1–20 μl) of samples (Jenkins *et al.*, 1988). Furthermore, the continuous response of an electrode system allows for on-line control, and the equipment required for electrochemical analysis is simple and cheap compared to most other analytical techniques. Depending upon the electrochemical property to be measured by a detector system, electrochemical biosensors may further be divided into conductometric, potentiometric, and amperometric biosensors.

2.4.1 Conductometric Biosensors

Conductometric biosensors measure the changes in the conductance of the biological component arising between a pair of metal electrodes (Gerard *et al.*, 2002). Microbial metabolism usually results in an increase in both, conductance and capacitance, causing a decrease in impedence. Therefore, the concept of conductance, capacitance, impedence, and resistance are different ways of monitoring the test system and are all inter-related (Silley and Forsythe, 1996; Milner *et al.*, 1998). DeSilva *et al.* (1995) detected *Staphylococcus* enterotoxin B (SEB) and its specific antibody using an impedance based

sensor with an ultra-thin platinum film. The magnitude of the impedance (Z) decrease followed a simple relationship with SEB concentration in the range between 0.389 and 10.70 ng/ml SEB. The biosensor was specific to SEB as no irreversible impedance decreases occurred when the sensor was exposed to 100 ng/ml of kappa-casein, alpha-casein, or alpha-lactalbumin, in PBS.

Another conductometric biosensor was developed with a phthalocyanine thin film as a sensitive element for the detection of peroxidase labeled antibodies in aqueous medium (Sergeyeva *et al.*, 1998). With the developed sensor, concentrations of IgG in the range of 0.2-2 μg/ml were measured in a competitive mode with satisfactory accuracy. This performance was a slight improvement over a polyaniline-based conductometric biosensor developed previously for IgG detection (Sergeyeva *et al.*, 1996). The lowest antigen concentration that could be detected in the competitive electroimmunoassay was about 0.5 μg/ml. More recently, a conductometric biosensor for detecting foodborne pathogens was developed by Muhammad-Tahir and Alocilja (2003a; 2005a). The biosensor consisted of a lateral flow-based immunosensor and a reader for signal measurement. The lower limit of detection with *E. coli* O157:H7 and *Salmonella* Typhimurium was approximately 79 CFU/ml within 10 min.

2.4.2 Potentiometric Biosensors

Potentiometric measurements involve determination of the potential difference between an indicator and a reference electrode. They function under equilibrium conditions and monitor the accumulation of charge, at zero current, created by selective binding at the electrode surface (Koncki *et al.*, 2000). The transducer may be an ion-selective electrode (ISE) which is an electrochemical sensor based on thin films or

selective membranes as recognition elements. For example, ISE can detect ions such as fluoride, iodide, or gas (CO_2, NH_3) in complex biological matrices by sensing changes in electrode potential when the ions bind to an appropriate ion exchange membrane. The potential difference between these indicators and reference electrodes are proportional to the algorithm of the ion activity or gas concentration, as described by the Nernst equation:

$$\Delta E = \Delta E^0 - \frac{RT}{nF} \ln \frac{[C]^c[D]^d}{[A]^a[B]^b}$$, where ΔE = potential difference, R = gas constant (8.314 J mol^{-1} K^{-1}), T = Temperature (in °K), F = Faraday's constant, and A, B, C, and D are the reaction products for (aA + bB = cC + dD).

An ion-channel biosensor based on supported bilayer lipid membranes (BLM) has been reported for the rapid detection of *Campylobacter* species (Ivnitski *et al.*, 2000). The sensing element was a stainless-steel working electrode that was covered by an artificial BLM. Antibodies to bacteria embedded in the BLM were used as channel-forming proteins, and it was found that the total number of univalent ions, related to signal amplification, flowing through the channels was 10^{10} ions/s. The biosensor showed very good sensitivity and selectivity to *Campylobacter* species.

The recently developed light addressable potentiometric sensor (LAPS) based on field effect transistor (FET) technology has proved to be highly successful for detection of bacteria (Gehring *et al.*, 1998; Dill *et al.*, 1999b; Lee *et al.*, 2000; Tu *et al.*, 2000; Ercole *et al.*, 2002; Tu *et al.*, 2002; Ercole *et al.*, 2003; Hu *et al.*, 2004). An LAPS consists of n-type silicon doped with phosphorous and an insulating layer in contact with an aqueous solution where the immunoreaction takes place. The difference between the charge distribution at the surface of the insulating layer and a FET is used to detect

changes in the potential at the silicon-insulator interface. A LAPS measures an alternating photocurrent generated when a light source, such as a light emitting diode (LED) flashes rapidly. The photocurrent can only be measured on these discrete zones where the sensor is illuminated. Thus, LAPS may measure local changes by multiplexing the LED, and consequently, measure different analytes simultaneously using a single sensor (Owicki *et al.*, 1994).

Ercole *et al.* (2002) developed a biosensor for *E. coli* in drinking water based on a potentiometric alternating biosensing (PAB) transducer. The transducer principle based on LAPS, which revealed the production of ammonia by an urease - *E. coli* antibody conjugate, could be used to detect 10 CFU/ml in 1.5 h. Although FET-based devices offer improvements to potentiometric monitoring of bacteria, there are problems associated with these devices, such as light sensitivity of the materials used in their construction, poor reproducibility, and selectivity.

2.4.3 Amperometric Biosensors

Almost all microorganisms can be sensed amperometrically by their enzyme-catalyzed electro-oxidation/electro-reduction or their involvement in a bioaffinity reaction. Amperometry is based on the measurement of the current resulting from the electrochemical oxidation or reduction of an electroactive species at a constant applied potential. It is usually performed by maintaining a constant-potential at a working electrode or an array of electrodes with respect to a reference electrode. Ag/AgCl is the most common reference electrode. Suitable electrode materials for amperometry are noble metals, graphite, modified forms of carbon (carbon paste, glassy carbon, pyrolytic graphite), and conducting polymers. Amperometric biosensors have the advantage of being highly sensitive, rapid, and inexpensive (Ghindilis *et al.*, 1998).

Electrochemical measurements at electrode interfaces are easier to execute in very small volumes compared to optical measurements. Interest in miniaturized electrochemical biosensors, and the development of inexpensive and disposable sensors, has led to the application of thick- and thin-film technology in the manufacturing of biosensors. Amperometric systems have a linear concentration dependence compared to a logarithmic relationship in potentiometric systems. This makes amperometric immunosensors well suited for bacterial assay (Shah and Wilkins, 2003). Several amperometric immunosensors aimed at microbial detection using different biological sensing elements have been reported (Mirhabibollahi *et al.*, 1990; Nakamura *et al.*, 1991; Brooks *et al.*, 1992; Kim *et al.*, 1995; Rishpon and Ivnitski, 1997). Nakamura *et al.* (1991) developed an electrode system consisting of a basal-plane pyrolytic graphite (BPG) electrode and a porous nitrocellulose membrane filter to trap bacteria for the detection of bacteria in urine. The peak current of a cyclic voltammogram increased with increasing initial cell concentration of *E. coli* in urine from $5x10^2$ to $5x10^5$ cells/ml. Enzyme-linked amperometric immunosensors have also been developed for the detection of *S. aureus* and *Salmonella* (Brooks *et al.*, 1990; Mirhabibollahi *et al.*, 1990; Brooks *et al.*, 1992). This immunosensor could only detect 10^4–10^5 CFU/ml of *S. aureus*. A modified approach, incorporating an enzyme amplification step and amperometric detection of reduced mediator (ferrocyanide), was used to detect low numbers (1–5 CFU/g or per ml) of *Salmonella* in food after non-selective (18 h) and selective (22 h) enrichment steps.

Recently, a technique based on the amperometric quantification of total coliforms, along with the specific detection of *E. coli*, was reported (Mittelmann *et al.*, 2002). An

amperometric enzyme biosensor based on the electrochemical detection of β-galactosidase activity, using p-aminophenyl-β-D-galactopyranoside as substrate, was used to determine coliform bacteria. The specific detection of *E. coli* was by an antibody-coated electrode that binds to the target bacteria. Detection of 10^3 CFU/ml was carried out amperometrically (by screen-printed electrodes) in about 1 h, with a 100-fold increase in the sensitivity after 5-6 h of preenrichment.

Immunomagnetic beads have been applied in immunoelectrochemical assays for the detection of *S.* Typhimurium (Gehring *et al.*, 1996). This technique combined the selectivity of antibody-coated superparamagnetic beads with the rapidity and sensitivity of electrochemical detection of bacteria. *Salmonella* Typhimurium was sandwiched between antibody-coated magnetic beads and an enzyme-conjugated antibody. With the aid of a magnet, the beads were then localized onto the surface of disposable graphite ink electrodes in a multi-well plate format. After magnetic separation, the liquid was removed by aspiration, and 200 μl of p-aminophenylphosphate was added to the electrochemical cell, and p-aminophenol, the product of the enzymatic reaction, was measured using square wave voltammetry. Using this technique, a minimum of $8x10^3$ cells/ml of *S.* Typhimurium in buffer was detected in 80 min. These immunoassays are labor intensive and time-consuming due to the many washing steps, requirement of complex instrumentation for their automation, and great lack of sensitivity (Gehring *et al.*, 1996).

An amperometric detector for *E. coli* using self-assembled monolayers (SAM) and microelectromechanical systems (MEMS) was also developed (Gau *et al.*, 2001). A detector array was fabricated by MEMS such that multiple electrodes were deposited on a

silicon wafer. By the use of biotinylated thiol SAM on gold, a monolayer of streptavidin was immobilized on to a gold electrode surface to capture rRNA from *E. coli*. A high specificity was achieved using single-stranded (ss) DNA-rRNA hybridization. The sensor system could detect 10^3 *E. coli* cells without polymerase chain reaction.

2.4.4 Cyclic Voltammetry and DNA Biosensors

Electrochemical detection of DNA hybridization usually involves monitoring of a current response, resulting from the Watson–Crick base-pair recognition event, under controlled potential conditions (Okahata *et al.*, 1992; Wang, 1999). The probe-coated electrode is commonly immersed into a solution of a target DNA whose nucleotide sequence is to be tested. When the target DNA contains a sequence that matches the immobilized oligonucleotide probe DNA, a hybrid duplex DNA is formed at the electrode surface. These hybridization events are commonly detected via the increased current signal of an electroactive indicator (that preferentially binds to the DNA duplex), in connection to the use of enzyme labels or redox labels (Wang, 1999). A detection technique that has not been widely investigated is the electrochemical detection of sequence-specific DNA hybridization based on the intrinsic DNA signals (due to the electroactivity of the nucleic acid bases), which are influenced by the DNA structure. The research described herein will employ cyclic voltammetry, a potentiometric technique, to detect these intrinsic changes in DNA signals resulting from DNA hybridization. This will be achieved indirectly by measuring the changes in cyclic voltammogram profiles of a redox chemical (potassium hexacyanoferrate (II) trihydrate, $K_4Fe(CN)_6$) occurring due to DNA probe immobilization on the biosensor platform as well as target DNA hybridization.

Cyclic voltammetry, referred to as the electrochemical equivalent of spectroscopy, applies a triangular potential waveform to the system under investigation resulting in a current response (Figure 2-4). Cyclic voltammetry varies the potential to obtain information about the redox process of the system and to obtain the values of the redox potentials. A single voltage is reversed at some time after the electroactive species reacts, and the reverse sweep is able to detect any electroactive products generated by the forward sweep. The initial voltage at which the voltammogram is started is referred to as the rest potential (E_{rp}). Rest potential is the open-circuit voltage between the working and reference electrodes. Since this voltage varies linearly with time, the slope is referred to as the scan rate (V/s). A typical cyclic voltammogram for a reversible process is shown in Figure 2-5. The potential is plotted on the x-axis with the measured current values plotted on the y-axis. The values of interest on a cyclic voltammogram are the two peak currents and the corresponding potentials. The cathodic peak current, I_{pc}, and the anodic peak current, I_{pa}, occur at the reduction point and oxidation point, respectively (Robinson, 1987).

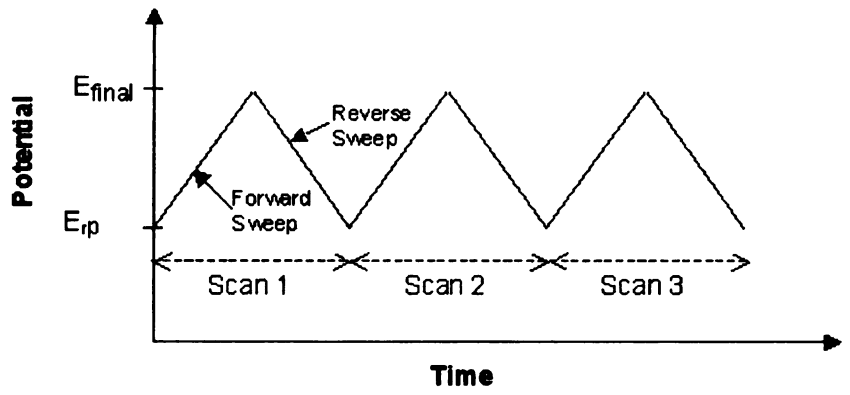

Figure 2-4. Excitation waveform for a cyclic voltammetry experiment (Adapted from (Robinson, 1987)).

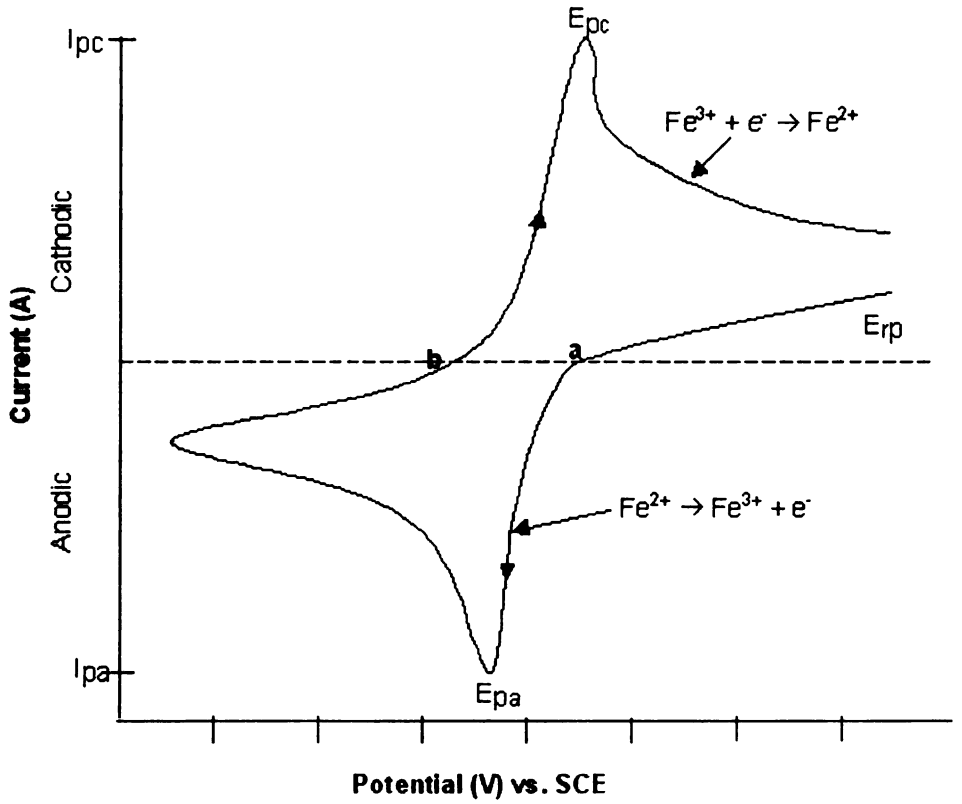

Figure 2-5. Typical current response for a reversible couple obtained with cyclic voltammetry (Adapted from (Robinson, 1987)).

The current is measured in a three-electrode electrochemical cell discussed further in Chapter 4: Section 4.3.4.8. The schematic representation of a three-electrode potentiostat is shown in Figure 2-6. In a potentiostat, the current is supplied through the counter electrode and is measured through the working electrode. The response of the working electrode depends on the concentration and composition of the analyte in question. The third electrode is a reference electrode that provides a constant reference potential against which the working electrode measures the output current. The reference electrode draws little or no current from the system and is unresponsive to the composition of the species under measurement (Robinson, 1987).

Cyclic voltammetry measures the faradaic current resulting from electron transfer. Two factors can affect the faradaic current: the rate the redox species diffuses to the electrode, and the rate of electron transfer. The rate of electron transfer for the common redox couple $Fe(CN)_6^{3-/4-}$ is reasonably fast. The reaction at the working electrode or cathode is $Fe(CN)_6^{3-} + e^- \leftrightarrow Fe(CN)_6^{4-}$, where one electron is added to the ferricyanide anion to reduce iron from the +3 to the +2 oxidation state. This reaction proceeds in both directions (cyclic process) so that species can be oxidized to $Fe(CN)_6^{3-}$, then reduced back to $Fe(CN)_6^{4-}$.

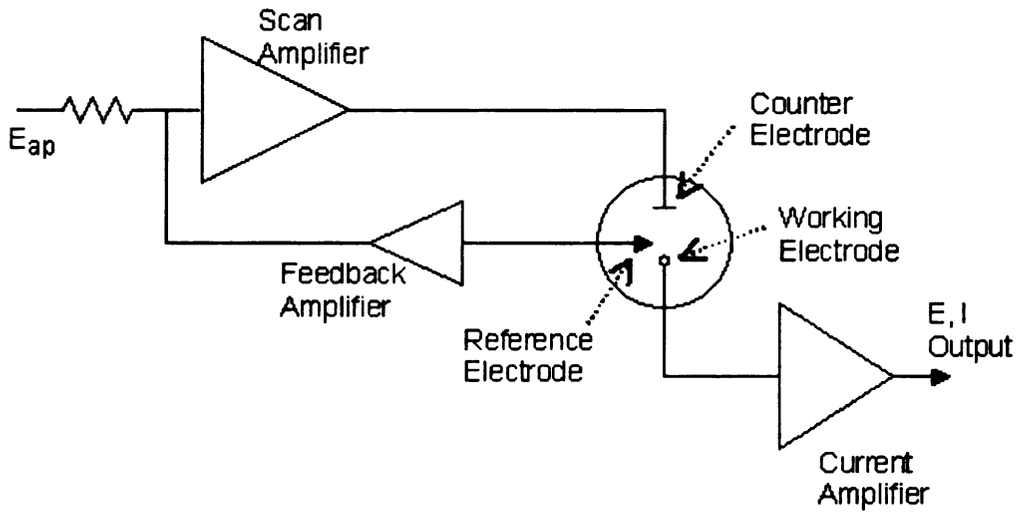

Figure 2-6. Schematic of a three-electrode potentiostat for cyclic voltammetry (Adapted from (Wang, 2000)).

During the scan, the applied potential becomes sufficiently positive at point a (Figure 2-5 above) to cause oxidation of Fe^{2+} to occur at the electrode surface. This oxidation is accompanied by anodic current, which increases rapidly until the surface concentration of Fe^{2+} approaches zero, as signaled by peaking of the current at point (I_{pa},E_{pa}) in Figure 2-5. The current then decays as the solution surrounding the electrode is depleted of Fe^{2+} due to its oxidation to Fe^{3+}. The magnitude of the current is described by the following equation: $I_t = nFAD_0(\frac{\partial C_0}{\partial X})_{x=0,t}$

where: I_t = Current at time, t (Amps).

n = Number of electrons, eq/mole.

F = Faraday's constant; 96,485 e/eq.

A = Electrode area, cm^2.

C = Concentration of oxidized species, mol/cm^3.

D_o = Diffusion coefficient of oxidized species, cm^2/s.

t = Time (s).

X = Distance from the electrode (cm).

The product $D_o (\partial C_o/\partial X)$ at x = 0,t is the flux or the number of moles of oxidized species diffusing per unit time to unit area of the electrode in units of mol/cm^2 s (Shippy and Burns, 2002).

During the positive scan in which Fe^{2+} is oxidized to Fe^{3+}, the depletion of Fe^{2+} in the vicinity of the electrode is accompanied by an accumulation of Fe^{3+}. After the direction of the potential scan is switched to a negative scan (reverse sweep), oxidation continues, as seen in Figure 2-4 above, until the applied potential becomes sufficiently negative to cause reduction of the accumulated Fe^{3+} (point b). Reduction of Fe^{3+} is signaled by the appearance of cathodic current. Once again, the current increases as the potential becomes increasingly negative until all of the Fe^{3+} near the electrode is reduced. When the concentration of Fe^{3+} is significantly depleted, the current peaks, and then decreases. Thus, in the forward scan Fe^{3+} is electrochemically generated, as indicated by the anodic current. In the reverse scan, the Fe^{3+} is reduced back to Fe^{2+}, as indicated by the cathodic current. The important parameters of cyclic voltammetry are the magnitude of the peak currents, I_{pa} and I_{pc}, and the potentials at which peaks occur, E_{pa} and E_{pc} (Shippy and Burns, 2002).

The current research will investigate the use of cyclic voltammetry to detect DNA hybridization under the hypothesis that the parameter, diffusion coefficient D_o, will be affected by the DNA probe immobilization and hybridization processes taking place on the biosensor platform surface, in turn influencing the output current, I_t, at any given time.

2.5 NANO-TUBULAR SILICON (NTS)

2.5.1 Silicon (Si)

Silicon (Si) was discovered by Jöns Jacob Berzelius, a Swedish chemist, in 1824 by heating chips of potassium in a silica container and then carefully washing away the residual by-products. Silicon compounds are the most significant component of the Earth's crust. Silicon is recovered from an abundant resource, sand. Most pure sand is quartz, silicon dioxide (SiO_2). Since sand is plentiful, easy to mine, and relatively easy to process, it is the primary ore source of silicon. Some Si is also retrieved from two other silicate minerals, talc and mica. The metamorphic rock, quartzite, is another source (quartzite is metamorphosed sandstone).

Two allotropes of Si exist at room temperature: amorphous and crystalline. The amorphous form appears as a brown powder, while crystalline silicon has a metallic luster and a grayish color. Single crystals of crystalline silicon can be grown with a process known as the Czochralski process. These crystals, when doped with elements such as boron, gallium, germanium, phosphorus or arsenic, are used to manufacture solid-state electronic devices, such as transistors, solar cells, rectifiers, and microchips (Gagnon, 2005). Silicon is a semi-conductor. When silicon is doped with dopants, such as boron and gallium that make it conductive by providing more holes than electrons, it is referred to as p-type Si. Conversely, silicon doped with dopants, such as phosphorus and arsenic that make it conductive by providing more electrons than holes, is termed n-type Si.

2.5.2 Silicon Etching

The etching of silicon surfaces in fluoride solutions is of both technological and fundamental significance. It is essential to semiconductor processing in integrated circuit

manufacture. It can also create materials with different properties. Based on the etching conditions, either perfectly flat H-terminated surfaces or H-terminated NTS is produced. Flat, clean Si surfaces are required for better device performance. NTS is of technological interest not only for its optical properties (Naftel *et al.*, 2000; Nguyen *et al.*, 2000; Setzu *et al.*, 2000), but also as a biomaterial (Wolf *et al.*, 1998), sensor (Lin *et al.*, 1997; Dancil *et al.*, 1999; Fichera *et al.*, 2003; Martin-Palma *et al.*, 2004), and substrate for laser desorption/ionization mass spectrometry (Shen *et al.*, 2001). Silicon is the only material that spontaneously gives rise to well defined tubules with nm dimensions. Other materials such as silicon carbide (SiC), gallium arsenide (GaAs), gallium phosphate (GaP), and indium phosphate (InP) can be made porous but with great difficulty. NTS is typically obtained by anodization in aqueous or ethanoic hydrofluoric acid solution (HF). The versatility of fluoride etching of Si has prompted research to elucidate the mechanisms behind the chemical, electrochemical, and laser assisted chemistry at the fluoride/Si interface (Cullis *et al.*, 1997; Chazalviel *et al.*, 2000; Jungblut *et al.*, 2002; Safi *et al.*, 2002).

A typical J-V curve is shown in Figure 2-7 for a diluted aqueous HF solution. Tubule formation occurs in the initial rising part of the curve for $0 < V < V_{ep}$. V_{ep} is the potential of the small sharp peak corresponding to the current J_{ep}. This peak is called the electropolishing peak. J_{ep} depends on the solution composition but not so much on the silicon substrate itself. When $V > V_{ep}$, electropolishing occurs since the surface becomes covered by an oxide layer, whose composition and dielectric properties depend on the applied potential. This provides a flat, clean Si surface useful for integrated circuits fabrication (Allongue, 1997).

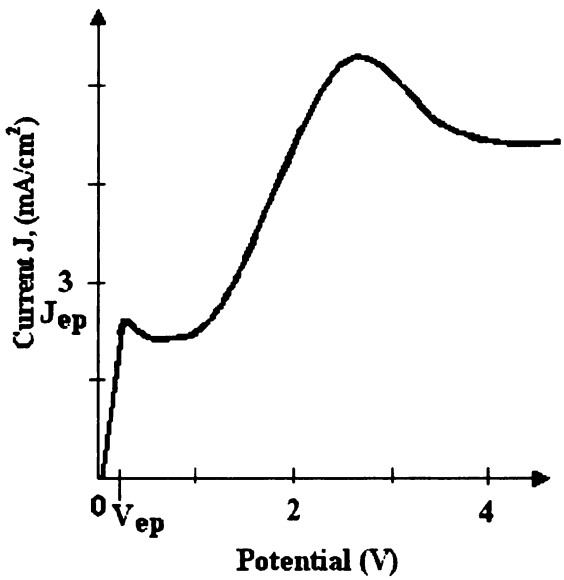

Figure 2-7. Typical J-V curve of p^+-Si in dilute aqueous HF solution. Nano-tubular Si is obtainable for $J < J_{ep}$. (Adapted from (Allongue, 1997))

The dissolution of p-type silicon and formation of NTS may be described by the reaction model in Figure 2-8 (Allongue, 1997). As long as the voltage is not raised above the electropolishing range ($J < J_{ep}$, so as to keep the supply of holes within a certain limit), steps A-B remain slower than steps C-E, and the surface remains H-terminated throughout etching (Chazalviel *et al.*, 2000; Safi *et al.*, 2002).

Etching is initiated by hole capture at the surface (Gerischer and Lubke, 1987, 1988; Gerischer *et al.*, 1993). For each silicon atom, dissolved, two holes are consumed in steps A and B. One molecule of hydrogen (step E) and two protons (steps B and C) are also generated on the surface. Once etching is initiated by hole capture at the surface, the ensuing reactions proceed rapidly to dissolve the activated Si atom. After the initial substitution steps Si-H → Si-OH → Si-F (steps A to C), undissociated HF and H_2O molecules chemically attack the strongly polarized $Si^{-\delta}$-$Si^{+\delta}$ back bonds, leaving the H

atom attached to the $Si^{-\delta}$ atom on the surface (steps D). The final step occurs in solution where the Si complex is further hydrolyzed with production of molecular H_2 (step E). Under most conditions, the hole capture step is the rate-determining step (RDS). Rate changes in response to solution composition changes must, therefore, be caused by one of the subsequent reactive steps. As the rate of substitution Si-H → Si-OH increases, by increasing the rate of hole supply, the density of Si-OH bonds increases and becomes such that the neighboring groups start to condense into Si-O-Si bridges. This is the early stage of oxide formation that corresponds to electropolishing (Allongue, 1997).

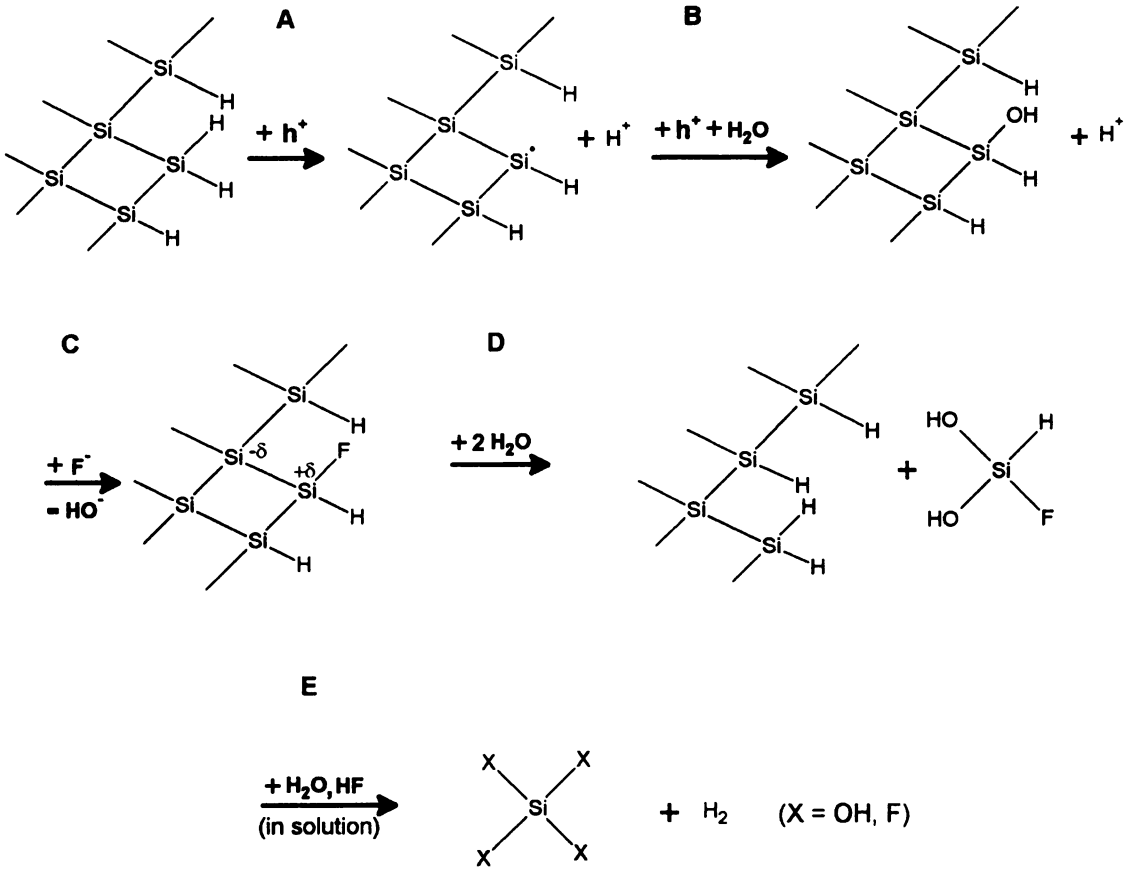

Figure 2-8. Reaction model for dissolution of nano-tubular silicon in aqueous HF solutions.

Theories that species, such as $(HF)_2$ and $(HF)_2^-$, apart from F^{-1} and HF have played an important role in the etch mechanism have not yet been proven. In particular, consensus is that $(HF)_2$ is not formed in aqueous solutions of HF, and consequently, any mechanism relying upon this species is incorrect. Species such as H_2O, OH^-, H^+, and F^- are involved in the faster reaction steps (Figure 2-8) (Kolasinski, 2003).

2.5.3 Nano-Tubular Si Porosity and Thickness Measurement

The porosity and thickness of the porous silicon layers are the most important parameters used to characterize porous silicon. The porosity is defined as the void within the porous silicon layer, and can be determined by weight measurements. The virgin wafer is first weighed before anodization (m_1), then just after anodization etching (m_2), and finally after complete dissolution of the porous layer in a 1 M NaOH aqueous solution (m_3). The porosity is given by the following equation:

$$P(\%) = \frac{(m_1 - m_2)}{(m_1 - m_3)}$$

From the above measurements, the thickness of the porous silicon layer can be determined as follows: $W = \frac{(m_1 - m_3)}{S \times d}$; where d is the bulk silicon density, and S is the area of the wafer exposed to HF during anodization (Allongue, 1997).

2.5.4 Nano-Tubular Silicon and Biosensors

Nano-tubular Si (NTS) has recently been investigated as a potential platform for biosensor applications. NTS has advantages over planar platforms in biosensor development due to the increased surface area, which will allow for higher sensitivity while using a smaller device. NTS can be easily synthesized directly from the same single-crystal silicon wafers used in the microelectronics industry, making it ideal for a

silicon-based optoelectronic technology (the combination of optical measurement and silicon microelectronics). An NTS-based device is compatible with established solid-state fabrication technologies.

Much of the developmental research has been related to the changes in the electronic and optical properties of NTS produced by quantum confinement. For example, light emitting devices (Hirschman *et al.*, 1996), photodetectors (Zheng *et al.*, 1992), and wave guides (Loni *et al.*, 1996) have been reported. Recently, novel sensor applications of NTS have also been developed, taking advantage of the high internal surface area (Lin *et al.*, 1997). Most of the recent developments in NTS sensors carry out a deposition step to fill the pores with metallic, dielectric or semiconducting oxide, enzyme, or molecular receptor films. This process has resulted in NTS sensors capable of detecting penicillin, alkali metal ions, humidity, and hydrocarbons (Stewart and Buriak, 2000).

2.5.4.1 Enzyme-based NTS biosensors

In the last five years, NTS has been used as a biosensor platform for enzyme-based biosensors. EIS (electrolyte-insulator-semiconductor) structures of nano-tubular n-$Si/SiO_2/Si_3N_4$ were fabricated to develop a capacitive pH sensor (Schöning *et al.*, 2000) using photolithography-based etching. The average pH sensitivity of the NTS pH microsensor was in the concentration range between pH 4 and pH 8, with the electrodes showing a potential stability of more than 1 week in the long term. Further research (Lüth *et al.*, 2000) showed that the enlargement of the active sensor area increased the measured capacitance values up to a factor of 30.

When used for glucose monitoring, the use of NTS as an efficient surface enlarging carrier matrix for immobilized enzymes was demonstrated. An increase in glucose turn-over of approximately 220 times as compared to an enzyme activated polished surface

was recorded. Glucose monitoring response was linear around 15 mM glucose with a satisfactory storage and operational stability (Bengtsson *et al.*, 2000b). Fichera *et al.* (2003) also demonstrated Si-based micro-biosensors for glucose detection in water solutions using NTS as the surface to bind the glucose oxidase enzyme. Micro channels localised below a suspended and auto-supporting NTS membrane, fabricated using surface micromachining processes, allowed the buffer solution to flow through the porous matrix. Photoluminescence, absorbance, and optical microscopy measurements were used to demonstrate biosensor functionality. In a similar study, DeLouise and Miller (2005) used NTS to gain a quantitative understanding of the effects of immobilization on enzyme activity. The activity of glutathione-S-transferase immobilized in electrochemically etched NTS films was compared with the enzyme in solution. The specific activity of the free enzyme in solution was observed to be four times higher than the immobilized enzyme, indicating that 25% of the enzyme was bound with the catalytic site in an inactive conformation or in a hindered orientation. To date, no studies have been reported employing NTS as a biosensor platform for bacterial detection with enzyme-based transduction.

2.5.4.2 Antibody-based NTS biosensors

Tinsley-Bown *et al.* (2000) used NTS as an optical interferometric biosensor with anti-rabbit IgG and the analyte rabbit IgG conjugated to horseradish peroxidase (HRP). Later, NTS multilayer stacks were developed for use as interference filters in the visible range (Martin-Palma *et al.*, 2004). The possibility of using these structures as biosensors was explored, based on the significant changes in the reflectance spectra before and after exposing the NTS multilayer to antibodies. Significant reduction of reflectance from NTS structures was observed when the sensor was exposed to polyclonal mouse antibodies.

The authors claim the possibility of developing biosensors based on the variation of the shape and/or position of the optical or photoluminescent spectrum from NTS. Further studies have not been reported to demonstrate the use of NTS as an optical interference filter-based biosensor.

Other biosensors developed with antibody-based detection have been demonstrated on planar silicon surfaces only. These include an ELISA (HRP)-based detection of thiabendazole (Flounders *et al.*, 1995), an evanescent field-based fluorescence detection of rabit IgG (Hofmann *et al.*, 2002), an ion-sensitive field effect transistor (ISFET)-based immunosensor to detect β-Bungarotoxin (Selvanayagam *et al.*, 2002), and a multi-analyte detection system using a protein biochip sensor array based on a complementary metal oxide silicon (CMOS) integrated circuit (Moreno-Bondi *et al.*, 2003).

2.5.4.3 DNA-based NTS biosensors

A DNA biosensor was developed based on induced wavelength shifts in the Fabry-Perot fringes in the visible-light reflection spectrum of appropriately derivatized thin films of NTS semiconductors. Binding of molecules induced changes in the refractive index of the NTS. The validity and sensitivity of the system was demonstrated for small organic molecules (biotin and digoxigenin), and 16-nucleotide DNA oligomers, at picomolar analyte concentrations (Lin *et al.*, 1997). Another DNA-based optical biosensor device was fabricated from a microcavity resonator made of various NTS layers. NTS containing silicon nanocrystals that could luminesce efficiently in the visible range was inserted between two Bragg reflectors (also made of NTS), to observe multiple and very narrow luminescence peaks. The position of these peaks was extremely sensitive to a small change in refractive index, such as that obtained when a biological

element was attached to the large internal surface of porous silicon. The authors successfully demonstrated a DNA hybridization detection sensor. Further progress was made with the DNA biosensor to detect viral DNA (Chan *et al.*, 2000; Chan *et al.*, 2001). Change in electrical properties (capacitance and conductance) of NTS layers upon exposure to organic solvents and water have also been investigated for application in biosensors (Archer *et al.*, 2003).

The use of NTS as a biosensor platform for detecting foodborne pathogens in conjunction with enzyme- and electrochemical transduction-based methods, such as cyclic voltammetry, has not been reported. In the development of a sensor, selectivity/specificity is an important issue. The sensor must be able to distinguish the element/target being detected from other non-target elements at the appropriate concentration for the target.

This research will develop a fabrication process to maximize the surface area of the biosensor platform to provide more reaction sites for the biological detection event. The NTS platform will be funtionalized into enzyme-, antibody-, and DNA-based biosensors, and used for detection of different bacterial targets. If appropriate transduction techniques are employed, such a biosensor platform will help achieve greater sensitivity while also improving the possibility of reducing the size of the sensor into a MEMS or nano-electromechanical system (NEMS) device. Specificity will be achieved by using enzymes, antibodies, and DNA probes specific to the target of interest, whenever possible. The versatility of the NTS platform will be demonstrated by using three biological sensing elements targeting three different pathogens with two immobilization techniques and two transduction mechanisms.

2.6 COLIFORMS/*ESCHERICHIA COLI*

2.6.1 Introduction

Direct testing of pathogenic bacteria and their toxins in foods is not routinely performed for quality control purposes (except for *Salmonella enterica* and *Staphylococcus aureus*). Instead, microbiological criteria for food safety often use tests for indicator organisms that suggest the possibility of a microbial hazard. Fecal coliforms are used as indicator organisms in water, foods, and beverages, the presence of which could suggest the possibility of microbial hazard (spoilage/pathogenic organisms). Coliforms are Gram-negative, rod-shaped, facultatively anaerobic bacteria that produce gas from glucose (and other sugars), and ferment lactose to acid and gas within 48 h at 35°C.

Escherichia coli, originally known as *Bacterium coli* commune, was identified in 1885 by the German pediatrician, Theodor Escherich. *Escherichia coli* is widely distributed in the intestine of humans and warm-blooded animals, and is the predominant facultative anaerobe in the bowel and part of the essential intestinal flora that maintains the physiology of the healthy host (Neill *et al.*, 1994). *Escherichia coli* is a member of the family *Enterobacteriaceae* (Ewing, 1986), which includes many genera, including known pathogens such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not regarded as pathogens, they can be opportunistic pathogens that cause infections in immunocompromised hosts. There are also pathogenic strains of *E. coli* that when ingested, causes gastrointestinal illness in healthy humans (Feng *et al.*, 2002).

In 1892, Shardinger proposed the use of *E. coli* as an indicator of fecal contamination based on the premise that *E. coli* is abundant in human and animal feces, and not usually found in other niches. Furthermore, since *E. coli* could be easily detected

by its ability to ferment glucose (later changed to lactose), it was easier to isolate than known gastrointestinal pathogens. Hence, the presence of *E. coli* in food or water became accepted as indicative of recent fecal contamination and the possible presence of pathogens. Although the concept of using *E. coli* as an indirect indicator of health risk was sound, it was complicated in practice, due to the presence of other enteric bacteria like *Citrobacter, Klebsiella, and Enterobacter* that can also ferment lactose and are similar to *E. coli* in phenotypic characteristics, so that they are not easily distinguished. As a result, the term "coliform" was coined to describe this group of enteric bacteria. Coliform is not a taxonomic classification but rather a working definition used to describe a group of bacteria. In 1914, the U.S. Public Health Service adopted the enumeration of coliforms as a more convenient standard of sanitary significance (Feng *et al.*, 2002).

Although coliforms were easy to detect, their association with fecal contamination was questionable because some coliforms are found naturally in environmental samples (Caplenas and Kanarek, 1984). This led to the introduction of the fecal coliforms as an indicator of contamination. Fecal coliform is a subset of total coliforms that grows and ferments lactose at elevated incubation temperature, hence also referred to as thermotolerant coliforms. Fecal coliform analyses are done at 45.5°C for food testing, except for water, shellfish, and shellfish harvest water analyses, which use 44.5°C (Neufeld, 1984; Clesceri *et al.*, 1998). The fecal coliform group consists mostly of *E. coli* but some other enterics, such as *Klebsiella*, can also ferment lactose at these temperatures and therefore, be considered as fecal coliforms. The inclusion of *Klebsiella* spp. in the working definition of fecal coliforms diminished the correlation of this group with fecal

contamination. As a result, *E. coli* has reemerged as an indicator, partly facilitated by the introduction of newer methods that can rapidly identify *E. coli*.

In recent years, strains of pathogenic *E. coli* have emerged as a serious food-borne pathogen, and have been associated with numerous outbreaks of disease (Uyttendaele *et al.*, 1999; Scotter *et al.*, 2000). Enterohemorrhagic *E. coli* (EHEC) are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome. EHEC produce verotoxin or Shiga toxins (Stx). Only those serotypes of Stx-producing *E. coli* that have been clinically associated with HC are designated as EHEC. Of these, *E. coli* O157:H7 is the most often implicated in illness worldwide (Nataro and Kaper, 1998). The infectious dose for *E. coli* O157:H7 is estimated to be 10 - 100 cells; but no information is available for other EHEC serotypes. EHEC have been implicated in outbreaks resulting from the consumption of water (Swerdlow *et al.*, 1992), undercooked ground beef (Davis *et al.*, 1993), cold sandwiches (Karmali, 1989), unpasteurized apple juice (Centers for Disease Control and Prevention, 1996), and sprouts and vegetables (Como-Sabetti *et al.*, 1997; Itoh *et al.*, 1998). Raw milk and dairy products, such as pasteurized milk and yoghurt contaminated with *E. coli* O157:H7, have been the main cause of several outbreaks of milk-borne disease since the 1980s (Daly *et al.*, 2002). EHEC O157:H7 exhibit slow or no fermentation of sorbitol and do not have glucuronidase activity, making them phenotypically distinct from other *E. coli* (Feng and Weagant, 2002).

Enterotoxigenic *E. coli* (ETEC) is recognized as the causative agent of travelers' diarrhea and illness, characterized by watery diarrhea with little or no fever. Found commonly in under-developed countries, it has been implicated as the etiologic agent in

outbreaks due to consumption of soft cheeses, Mexican-style foods, raw vegetables, and water. The infective dose of ETEC for adults has been estimated to be at least 10^8 cells; but the young, the elderly, and the immunocompromised may be susceptible to lower levels (Feng and Weagant, 2002).

Enteroinvasive *E. coli* (EIEC) closely resemble *Shigella*, and cause an invasive, dysenteric form of diarrhea in humans. No known animal reservoirs, except for infected humans, exist for EIEC. Volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults. Unlike typical *E. coli*, EIEC are non-motile and do not ferment lactose. Enteropathogenic *E. coli* (EPEC) cause a profuse watery diarrheal disease. It is a leading cause of infantile diarrhea in developing countries. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Through volunteer feeding studies, the infectious dose of EPEC in healthy adults has been estimated to be 10^6 organisms (Feng and Weagant, 2002). Because of their high infectious dose, analysis for ETEC, EIEC, and EPEC are usually not performed unless high levels of *E. coli* have been found in a food.

2.6.2 Traditional Detection Methods

Almost all the methods used to detect *E. coli*, total coliforms (TC) or fecal coliforms (FC) are enumeration methods that are based on lactose fermentation. Detection of coliforms is used as an indicator of unsanitary quality of water or as a general indicator of unsanitary conditions in the food-processing environment. Fecal coliforms are the standard indicator of choice for shellfish and shellfish harvest waters; and *E. coli* is used to indicate recent fecal contamination or unsanitary processing (Downes and Ito, 2001). Traditionally, the detection of coliforms (fecal or total) and *E. coli* is accomplished using non-selective and selective enrichment of samples in broth and enumeration on solid agar

medium. The Most Probable Number (MPN) method is a statistical, multi-step assay consisting of presumptive, confirmed, and completed phases. The method consists of inoculating a series of tubes with appropriate decimal dilutions of the water sample. Production of gas, acid formation or abundant growth in the test tubes after 48 h of incubation at 35°C constitutes a positive presumptive reaction. All tubes with a positive presumptive reaction are subsequently subjected to a confirmation test. The formation of gas in a brilliant green lactose bile broth fermentation tube at any time within 48 h at 35°C constitutes a positive confirmation test. Typically, only the first 2 phases are performed in coliform and fecal coliform analysis, while an additional phase is performed for *E. coli*. The 3-tube MPN test is used for testing most foods (Food and Drug Administration, 2000). The 5-tube MPN is used for water, shellfish, and shellfish harvest water testing, and there is also a 10-tube MPN method that is used to test bottled water or samples that are not expected to be highly contaminated (Neufeld, 1984; Clesceri *et al.*, 1998). The three phases of the MPN procedure take five days to complete. There is also a solid medium plating method for coliforms that uses Violet Red Bile Agar, which contains neutral red pH indicator, so that lactose fermentation results in the formation of pink colonies. This procedure takes 2-3 days for confirmation.

There are also membrane filtration (MF) tests for TC and FC that measure aldehyde formation due to fermentation of lactose (Feng *et al.*, 2002). The MF technique is fully accepted and approved as a procedure for monitoring drinking water microbial quality in many countries. This method consists of filtering a water sample on a sterile filter with a 0.45-μm pore size that retains bacteria, incubating this filter on a selective medium, and enumerating typical colonies on the filter. Media and incubation conditions for the MF

method tested for optimal recovery of coliforms include m-Endo-type media (incubation 24 h at 35°C for TC) in North America, and the Tergitol-TTC medium (incubation for 24 and 48 h at 37°C and 44°C for TC and FC, respectively) in Europe (Clesceri *et al.*, 1998; Rompre *et al.*, 2002). Other media, such as MacConkey agar and the Teepol medium, have also been used. A significant advantage of the MF technique over the MPN method is that with MF, the examination of larger volumes of water is feasible, which leads to greater sensitivity and reliability. MF also offers a quantitative enumeration comparatively to the semiquantitative information given by the MPN method. However, the MF method takes 48 h to complete. While some raw foods would be expected to have some coliforms because of their association with the environment, the presence of *E. coli* in heat-processed foods means either process failure or post processing contamination from equipment, employees, or from contact with contaminated food and food products. Contamination of *E. coli* implies a risk that other enteric pathogens may be present in the food. Thus, a more rapid detection of TC, FC, and *E. coli* (compared to MPN and MF) is very important to the food industry for quality control purposes.

2.6.3 Rapid Detection Methods

Methods for rapid detection and enumeration of microorganisms on membrane filters based on either ATP-bioluminescence (Millipore, Bedford, MA) or esterase activity (Chemunex, Maisons-Alfort, France) with complementary instrument platforms have been commercialized (Raynolds and Fricker, 1999; Upperman *et al.*, 1999). These methods, however, do not provide identification of the bacterial species.

2.6.3.1 Antibody-based methods

Rapid immunological methods are based on the specific recognition between antibodies and antigens, and the high affinity that is characteristic of this recognition

reaction. Depending on the taxonomic level of the targeted antigens, immunological methods permit detection of antigens at family, genus, species, or serotype levels. Two types of antibodies can be produced: polyclonal and monoclonal antibodies, which are more specific to the target organism. The properties of the antigen–antibody complex can be used to perform an immunocapture of cells or antigens by enzyme-linked immunosorbent assay (IMS or ELISA), or to detect targeted cells by immunofluorescence assay (IFA) or immuno-enzyme assay (IEA). Obst *et al.* (1989) developed an ELISA using a monoclonal antibody against the enterobacterial common antigen (ECA), a lipopolysaccharide that is linked within the outer membrane of *Enterobacteriaceae*. This method was not sensitive enough, however, and so pre-enrichment of the sample in a selective broth for 24 h was necessary in order to reach the detection limit of the ELISA (10^6 cells/ml). Cross-reactivity with different strains of *Aeromonas*, which are important competitors to coliform bacteria growing on m-Endo media, as well as *Psedumonas* and *Bacillus*, was also observed (Hübner *et al.*, 1992). Rapid methods, using the ELISA and EIA techniques in conjunction with luminescence and amperometry, have been developed specifically for *E. coli* O6 and *E. coli* O157:H7 strains, respectively (Abdel-Hamid *et al.*, 1999a; Schalch and Stolle, 2000; Trevanich *et al.*, 2000b). The ELISA is a rapid, simple, and quite sensitive test, which allows the detection of less than 10^{-9} g of antigenic-protein (Stryer, 1988). However, assay limitations are often associated with the specificity of the antibody used, the concentration of both antibody and antigen, and the type of reaction solution used. In addition, the solid matrix used often leads to non-specific binding of the antigen or of the second antibody (Kfir and Genthe, 1993). ELISA application to the detection of specific cells from a naturally contaminated sample is

limited. High levels of non-targeted microflora and diverse materials associated with the sample may interfere on the level of the specificity of the ELISA method (Hanai *et al.*, 1997).

2.6.3.2 Molecular methods

Molecular and biochemical technologies have been developed to address the issues of both rapid and simultaneous detection, identification, and enumeration of microorganisms following membrane filtration. The biochemical methods utilize activities of cellular enzymes to detect individual cells (Van Poucke and Nelis, 2000a) or microcolonies following a short growth step (Sarhan and Foster, 1991; Bauters *et al.*, 1999; Van Poucke and Nelis, 2000b). DNA-based molecular methods target various gene fragments unique to the bacteria. Primers based on the *lacZ* gene have been used for the polymerase chain reaction (PCR)–based detection of coliforms because conventional coliform monitoring methods are based on the expression product (β-galactosidase) of this gene (Bej *et al.*, 1990; Bej *et al.*, 1991a; Bej *et al.*, 1991b; Fricker and Fricker, 1994). For the specific detection of *E. coli*, a region of the *malB* gene that codes for a maltose transport protein was the first target sequence proposed (Bej *et al.*, 1990). However, due to cross reactivity with members of the *Shigella* and *Salmonella* genera, the *uidA* gene has been used more extensively for the PCR-based detection of *E. coli* (Bej *et al.*, 1991a; Bej *et al.*, 1991b; Tsai *et al.*, 1993; Fricker and Fricker, 1994; Juck *et al.*, 1996; Iqbal *et al.*, 1997; Rodríguez and Alocilja, 2005). However, many food types contain PCR inhibitors which co-purify with the target DNA (González *et al.*, 1999), thereby requiring extensive sample preparation to remove, dilute or inactivate inhibitors prior to PCR amplification (Fratamico *et al.*, 2000). In many cases, the low level of *E. coli* in food

types requires a pre-enrichment step to overcome poor sensitivity, which increases the overall assay time by up to 24 h (Seo *et al.*, 1998; Scotter *et al.*, 2000).

Direct fluorescence in situ hybridization (FISH) methods based on fluorescently labeled DNA probes (Muruyama and Sunamura, 2000; Tottorello and Reineke, 2000), and indirect FISH methods using biotinylated peptide nucleic acid (PNA) probes (Prescott and Fricker, 1999), have been described for real-time analysis. FISH uses oligonucleotide probes to detect complementary nucleic acid sequences. This method exploits the ability of nucleic acids to anneal to one another in a very specific complementary way to form hybrids. PNA probes are DNA mimics with improved hybridization characteristics (Egholm *et al.*, 1993; Nielsen *et al.*, 1994), which have been exploited in variety of different molecular techniques (Thisted *et al.*, 1996; Just *et al.*, 1998; Taneja *et al.*, 2001) and used for identification of microorganisms (Stender *et al.*, 1999; Stender *et al.*, 2001a). The popularity of the FISH technique is due to its advantages over radioactive labeling, which include sensitivity, speed of visualization of single cells (by means of microscopy or cytometrical devices), stability of the hybridization products, safety, diminished detection time, multiple labels (multiple colors), and ease of use (Richardson *et al.*, 1991; DeLong, 1993; Swinger and Tucker, 1996). However, the practical applicability of FISH has been limited to samples with a high level of contamination as the results are interpreted by manual epifluorescence microscopy allowing only a small part of a membrane filter to be viewed within a reasonable time (Stender *et al.*, 2001b).

2.6.3.3 Enzyme-based assays

Luminescence-based enzymatic assays are finding increased use in the food industry (Vidon *et al.*, 1994; Giese, 1995; Trevanich *et al.*, 2000a; Vidon *et al.*, 2001) due to their

simplicity and application to a wide variety of foods. Based on the enzymatic properties of coliforms, a defined substrate method has been developed to overcome some limitations of the traditional MTF and MF techniques. Unlike these techniques, which eliminate the growth of non-coliform bacteria with inhibitory chemicals, the defined substrate technology is based on the principle that only the target microbes (TC and *E. coli*) are fed and no substrates are provided for other bacteria. A defined substrate is used as a vital nutrient source for the target microbe. During the process of substrate utilization, a chromogen or a fluorochrome is released from the defined substrate, indicating the presence of the target microorganisms. Recently, Lee and Denninger (Lee and Denninger, 1999) developed a technique utilizing Adenosine Tri Phosphate (ATP) luminescence. Although this technique is sensitive and shows correlation to culture techniques, the bacteria must be viable to be detected. Bacteria that have been starved or damaged may not react in this test.

Another method based on the enzyme β-D-glucuronidase produced by 94-96% of *E. coli*, which hydrolyses 4-methylumbelliferyl-β-D-glucuronide (MUG) to form a blue fluorescent product, has been developed (Venkateswaran *et al.*, 1996; Manafi, 2000). Members of the *Salmonella, Shigella, and* some *Yersinia* spp. produce β-D-glucuronidase as well, while *Staphylococcus* spp. can hydrolyze MUG, thus making the method only useful as a preliminary test for detection of *E. coli*. Moreover, a mutation in the *uid*A gene in *E. coli leads* to false-negative identification when using β-D-glucuronidase as a method of detection, while certain strains of *E. coli*, such as O157, fail to produce this enzyme (Yokoigawa *et al.*, 1999). Thus, a more widely found enzyme for detecting *E. coli* is necessary.

β-galactosidase (beta-D-galactoside galactohydrolase E.C. 3.2.1.23; lactase) is an enzyme produced by all strains of *E. coli* as a part of its regular metabolic process. The enzyme catalyzes the hydrolysis of lactose and many beta-D-galactopyranosides. The enzyme is encoded by the *LacZ* gene, which encodes a 116 kD polypeptide of 1023 amino acids in addition to the N-terminal f-met (Wallenfels and Weil, 1972). β-galactosidase catalyzes the following hydrolysis:

β-D-galactoside + H_2O → galactose + alcohol (Adhya, 1996).

β-galactosidase is widespread in microorganisms, animals, and plants. However, Tryland and Fiksdal (1998b) observed that the β-D-galactosidase-positive, noncoliform isolates exhibited a mean β-D-galactosidase activity of at least 2 log units less than for *E. coli*. The noncoliform bacteria must be present in correspondingly higher concentrations than those of target bacteria to interfere in a rapid assay for detection of coliform bacteria. In foods, *E. coli* is usually present in numbers higher than noncoliform organisms that produce β-galactosidase. Thus, the enzyme can be used as a marker to develop rapid methods for *E. coli* detection.

Thermally stable dioxetanes can be enzymatically "triggered" to produce chemiluminescence using β-galactosidase (Schaap *et al.*, 1991). The use of such technique has been reported (Van Poucke and Nelis, 1995, 1997). A useful chemiluminescent substrate for β-galactosidase is a phenyl galactose-substituted dioxetane (Schaap *et al.*, 1991). Lumi-Gal 530 (Lumigen, Inc., Southfield, MI), a commercial formulation of 4-methoxy-4-(3-β-D-galactosidephenyl)spiro[1,2-dioxetane-3,2'-adamantane], reacts with β-galactosidase (a 4-step process) to generate chemiluminescence, which decays with a half-life of 10 min at 37°C (Beale *et al.*, 1992).

Lumi-Gal 530 was shown to have a 20-fold greater sensitivity over other standard spectrophotometric assays when used with a pure β-galactosidase solution (Beale *et al.*, 1992). However, this dioxetane substrate was not evaluated any further to develop a rapid chemiluminescence-based detection assay for *E. coli*. In this research, Lumi-Gal 530 will be used to develop a single tube chemiluminescence-based assay for detection *E. coli*, which will then be used as the basis for development of a NTSlicon-based biosensor for *E. coli* detection.

2.7 *SALMONELLA*

2.7.1 Introduction

Salmonella is a rod-shaped, motile bacterium, except for *S. gallinarum* and *S. pullorum*, nonsporeforming and Gram-negative. *Salmonella* is ubiquitous in the natural environment, widespread in animals, especially in poultry and swine. Environmental sources of the organism include water, soil, insects, factory surfaces, kitchen surfaces, animal feces, raw meats, raw poultry, and raw seafoods (Center for Food Safety & Applied Nutrition, 1992). The genus *Salmonella* consists of resilient microorganisms that readily adapt to extreme environmental conditions. Some strains of *Salmonella* grow at temperatures as high as 54°C while also exhibiting ability to grow in refrigerated foods stored at 2 to 4°C. Salmonellae have the ability to proliferate at pH values ranging from 4.5 to 9.5, with a pH optimum for growth between 6.5 and 7.5. High salt concentration exerts a bacteriostatic effect by decreasing the water activity (a_w) leading to bacterial plasmolysis commensurate with the hypertonicity of the suspending medium. Foods with water activity of 0.93 or less do not support the growth of salmonellae. However, bacterial salt tolerance has been shown to increase at temperatures between 10 to 30°C. The growth or survival of this pathogen under extreme pH, temperature, and salt concentration conditions raises concerns of safety in foods, such as fermented products (e.g., sausages, fermented raw milk products), modified-atmosphere-packaged and vacuum-packaged foods that contain high levels of salt (D'Aoust, 1997).

Salmonella Typhimurium and *S.* Enteritidis are the most common *Salmonella* serotypes found in the United States. According to CDC, salmonellosis is the most common foodborne illness (CDC, 2002b). Over 40,000 actual cases are reported yearly in the U.S. (CDC, 2002a). Approximately 500 (Mead *et al.*, 1999) to 1,000 (CDC, 2001)

persons die annually from *Salmonella* infections in the U.S. The estimated annual cost of human illness caused by *Salmonella* is $3 billion (Mead *et al.*, 1999). *Salmonella* Enteritidis has frequently been observed as a contaminant in foods, such as fresh produce (De Roever, 1998), eggs and poultry products (Cohen *et al.*, 1994). While various *Salmonella* species have been isolated from the outside of egg shells, presence of *S.* Enteritidis inside the egg, in the yolk, is of great concern as it suggests vertical transmission, i.e., deposition of the organism in the yolk by an infected layer hen prior to shell deposition (Center for Food Safety & Applied Nutrition, 1992).

Human *Salmonella* infection can lead to enteric (typhoid) fever, enterocolitis, and systemic infections by non-typhoid microorganisms. Typhoid and paratyphoid strains are well adapted for invasion and survival within host tissues, causing enteric fever, a serious human disease. Non-typhoid *Salmonella* causes salmonellosis, which is manifested as gastroenteritis with diarrhea, fever, and abdominal cramps. Severe infection could lead to septicemia, urinary tract infection, and even death in at-risk populations (young, elderly, and immunocompromised individuals). Raw meats, poultry, eggs, milk and dairy products, fish, shrimp, frog legs, yeast, coconut, sauces and salad dressing, cake mixes, cream-filled desserts and toppings, dried gelatin, peanut butter, cocoa, and chocolate are some of the foods associated with *Salmonella* infection (D'Aoust, 1997).

All known strains (about 2400) of *Salmonella* are pathogenic with a very low infectious dose observed in some of the foodborne outbreaks traced back to *Salmonella* contamination. Newborns, infants, the elderly, and immunocompromised individuals are more susceptible to *Salmonella* infections than healthy adults (D'Aoust, 1997). The developing immune system in newborns and infants, the frequently weak and/or delayed

immunological responses in the elderly and debilitated persons, and low gastric acid production in infants and seniors facilitate the intestinal proliferation and systemic infection of salmonellae in this susceptible population (Blaser and Newman, 1982). Moreover, in recent years, concerns have been raised because many strains of *Salmonella* have become resistant to several of the antibiotics traditionally used to treat it, in both animals and humans, making *Salmonella* infections an important health concern in both developed and undeveloped countries. The majority of the increased incidence of resistance can be attributed to *Salmonella* Typhimurium DT104. Treatment of this infectious disease is complicated by its ability to acquire resistance to multiple antibiotics (Carlson *et al.*, 1999). Evidence suggesting that ingestion of only a few *Salmonella* cells can develop a variety of clinical conditions (including death) is a reminder for food producers, processors, and distributors that low levels of salmonellae in a finished food product can lead to serious public health consequences, and undermine the reputation and economic viability of the incriminated food manufacturer. Thus, early and rapid detection of *Salmonella* is very important to the food industry so that appropriate measures can be taken to eliminate infection through food.

2.7.2 Traditional Detection Methods

The method currently in use for *Salmonella* identification is a culture of the bacteria from the food samples, a time-consuming and laborious process. The conventional procedure of pre-enrichment broth, selective enrichment broth, and differential agar requires 3-5 days to achieve a presumptive identification of *Salmonella* bacteria. The biochemical identification of foodborne and clinical *Salmonella* species is generally coupled to serological confirmation, a complex and labor-intensive technique involving the agglutination of bacterial surface antigens with *Salmonella*-specific

antibodies. These include O lipopolysaccharides (LPS) on the external surface of the bacterial outer membrane, H antigens associated with peritrichous flagella, and the capsular (Vi) antigen. The diagnostic value of biochemical traits is generally combined with serological characterization, resistance to antibiotics, plasmid profiling, and phage-typing assays. The whole process for confirmation of *Salmonella* serovars can take as long as 7-10 days (Food and Drug Administration, 2000) and is likely to be replaced by molecular techniques.

2.7.3 Rapid Identification of *Salmonella*:

Several techniques for improving the detection of *Salmonella* serovars in food as well as feces, such as the use of a selective culture medium, enzyme-linked immunosorbent assay (ELISA), and DNA-based techniques, have been developed (Araj and Chugh, 1987; Aspinall *et al.*, 1992; Chiu and Ou, 1996; Amavisit *et al.*, 2001).

Valdivieso-Garcia *et al.* (2003) used a combination of the above enrichment in selective culture medium and ELISA to develop a bioluminescent enzyme immunoassay (BEIA). The study used streptavidin-biotinylated firefly luciferase complex as a reporter. *Salmonella* cultures were grown for 24 h. After enrichment, the total test time for the BEIA was 1.5 h. The results obtained in the study indicated a sensitivity of 10^5-10^6 CFU/ml.

The VITEK immunodiagnostic assay system (VIDAS) is an automated, qualitative, enzyme-linked fluorescent immunoassay method. Comparative studies with a variety of food types have found favorable correlation between conventional test methods and the VIDAS system (Blackburn *et al.*, 1994; Curiale *et al.*, 1997; De Medici *et al.*, 1998; Walker *et al.*, 2001). For *Salmonella* detection, the specific VIDAS reagent strip relies on immunocapture with specific antibodies raised against the O and H antigens of

the organism. A relative fluorescence value is determined for samples based on the measured fluorescence relative to background. This value is used for interpretation of results. Use of such a system has a number of advantages over conventional culture methods, including time to obtain a negative or presumptive positive result and technical ease of performing the test.

Problems remain with sample processing, sensitivity, and specificity that have limited the routine use of the above antibody-based procedures.

Polymerase chain reaction (PCR) technology, which allows amplification of a specific fragment of nucleic acid, has been used to identify the presence of specific pathogens directly from clinical as well as food specimens. PCR is an extremely sensitive test, able to amplify picogram quantities of DNA. Although the PCR assay is sensitive, it may be susceptible to false negative results when applied to food and clinical samples. This may be due to a variety of inhibitory substances such as chelating agents and food components (Widjojoatmodjo *et al.*, 1992). To overcome the problem of false negative reactions, inclusion of an internal positive control (IPC) (Chadwick *et al.*, 1998), and performing PCR after preincubation in an enrichment broth has been investigated for human (Chiu and Ou, 1996; Luk *et al.*, 1997; Marsiglia *et al.*, 1997; Lin and Tsen, 1999), animal (Stone *et al.*, 1994; Stone *et al.*, 1995; Cohen *et al.*, 1996), fecal (Widjojoatmodjo *et al.*, 1992), and food samples (Chen *et al.*, 1997; Bennett *et al.*, 1998; Rijpens *et al.*, 1999). This has been found as a useful and a more rapid method because the enrichment step increases the number of viable *Salmonella* in the sample, and therefore, the sensitivity of the assay (Gouws *et al.*, 1998). PCR methods and DNA extraction procedures for *Salmonella* have been described for human, animal, and food samples

(Soumet *et al.*, 1994). However, these extraction methods using lysozyme, proteinase K, and phenol-chloroform-isoamyl alcohol are expensive and time consuming, whilst methods using silica beads or glass particles to bind with DNA are more rapid but expensive (Boom *et al.*, 1990; Mahon and Lax, 1993; Tuchili *et al.*, 1996).

Recent advancements in PCR technology combined capillary thermocycling and real-time fluorescence detection, and/or quantification of the PCR product in different formats such as SYBR Green I dye binding (Rantakokko-Jalava and Jalava, 2001; Szuhai *et al.*, 2001; De-Medici *et al.*, 2003; Jothikumar *et al.*, 2003), and hybridization probe (Wittwer *et al.*, 1997; Bellin *et al.*, 2001; Eyigor and Carli, 2003). Carli *et al.* (2001) detected salmonellae in chicken feces by a tetrathionate broth (TTB) enrichment, capillary PCR, and capillary gel electrophoresis, and obtained positive results with high sensitivity. However, this process required separate units for capillary PCR and gel electrophoresis runs, and it was not real time. Eyigor *et al.* (2002) applied the real-time PCR technology with the SYBR Green I Dye format to TTB enrichment culture of a standard *Salmonella* isolation method from poultry. *Salmonella* was detected in 25 min from up to 32 18-h primary enrichment sample cultures from subclinically infected chickens. This PCR was about 10 times faster than the previously reported conventional PCR detection methods (Aabo *et al.*, 1995; Cohen *et al.*, 1996) and yielded more rapid results. The Light Cycler Real time PCR system's (Roche Diagnostics, Mannheim, Germany) hybridization probe design allows sequence-specific detection of the amplicon with the use of fluorescence resonance energy transfer between the two fluorophores used in the probes. Also, this system reduces the detection time and enables the process of up to 32 samples simultaneously.

However, the need for expensive equipment (that are usually not portable) and expertise required in performing PCR makes these DNA-based detection methods cost prohibitive and limits their application in the field. Biosensors can provide a viable solution to the need for cheap, portable, sensitive, and specific rapid detection methods for pathogens.

Specific and selective detection of *Salmonella* Typhymurium based on the use of a polyclonal antibody immobilized by the Langmuir–Blodgett method on the surface of a quartz crystal acoustic wave device was demonstrated in liquid samples (Olsen *et al.*, 2003). These biosensors were selective to *S.* Typhymurium at levels above 10^4-10^6 CFU/ml in the presence of large concentrations of *Escherichia coli* O157:H7.

A time-resolved fluoroimmunoassay was developed for the simultaneous detection of *E. coli* O157:H7, *S.* Typhimurium and *S.* Enteritidis in foods. Immunomagnetic beads were used to capture and concentrate the target pathogens. The captured bacteria were allowed to form sandwiched complexes with europium-labeled anti-*E. coli* O157 antibodies and/or samarium-labeled anti-*Salmonella* antibodies. The specific time-resolved fluorescence associated with highly fluorescent europium or samarium micellar complexes were measured to estimate the quantity of *E. coli* and *Salmonella*, respectively, with resulting sensitivity of 10^3-10^4 cfu/ml for pure cultures of the target pathogens (Shu *et al.*, 2002).

A Threshold Immunoassay System, using a light-addressable potentiometric sensor, was used to detect *S.* Typhimurium to levels as low as 119 CFUs (10-20 µl sample volume). This biosensor utilized solution-based binding of the biotin and fluorescein labeled antibodies to *Salmonella*, followed by filtration-capture of the

immunocomplex on a biotin-coated nitrocellulose membrane. Lastly, an anti-fluorescein urease conjugate was bound to the immunocomplex. Detection of the bound immunocomplex was made possible via a silicon chip-based light-addressable potentiometric sensor. In the presence of the urea, urease converted the substrate to ammonia and CO_2, resulting in a pH change at the silicon surfacethat was monitored with time, and reported in mV/s. Total assay time was about 2 h. Sample debris was found to impede the sample flow through the membrane and hence, the signal output (Dill *et al.*, 1999a).

Salmonella Enteritidis and *Listeria monocytogenes* were detected in real time using a surface plasmon resonance (SPR) biosensor. The respective antibodies against the target pathogens were immobilized on the gold sensor surface as a covalently crosslinked double-layer or covalently bound on a crosslinked albumin layer. The target-binding event was detected through prism excitation of surface plasmons and spectral interrogation. *Salmonella* and *Listeria* could only be detected by the sensor at concentrations of 10^6 CFU/ml (Koubova *et al.*, 2001a).

A rapid and sensitive primary screening method with a proper sampling plan is required to detect *Salmonella* in the food industry. While several biosensors are being developed for detection of *Salmonella*, very few biosensors are commercially available. With the recent advances in the silicon-based IC industry, biosensors using a silicon-based platform will be more amenable to commercialization using existing microfabrication processes. The current research will focus on developing a silicon-based biosensor with antibody and DNA probes as the biological recognition elements for *Salmonella enterica.*

CHAPTER 3: RESEARCH HIGHLIGHTS

3.1 INNOVATION AND NOVELTY

The innovation of this research was the use of nanotechnology (nano-tubular Si) combined with immuno-chemistry and molecular biology in the fabrication of a field-deployable nano-structured biosensor for food safety and quality assurance, health care, and bio-defense purposes. The research combined the use of chemiluminescence and electrochemical transduction mechanisms in a versatile NTS platform to build biosensors for detection of foodborne pathogens that were simple, easy to use (with no need for light source and filters), sensitive, and specific to the target. Three biological sensing elements (enzymes, antibodies, and nucleic acids) were used to functionalize the biosensors. The output light from the chemiluminescence transducer, measured using a photomultiplier tube (luminometer), was correlated with the number (cells) of the target being detected, thus indicating the presence or absence of the target, and in some cases, the number of target cells in the sample. Cyclic voltammetry was used to detect target DNA hybridization on the biosensor chip using intrinsic changes in DNA signals, a property not yet investigated in the literature for foodborne pathogen detection.

In the long-term, a portable handheld nucleic acid-based biosensor could be built in collaboration with commercial companies. The biosensor will have a chip with a removable cartridge and on-board circuitry for data acquisition. The cartridge will consist of different chambers for reagent storage, performing PCR (if that is found to be necessary step before detection), and the chemiluminescence pathogen detection system. The detection system will consist of multiple detection "spots" immobilized with probes specific to different targets of interest. Each of the spots will be individually addressable

electronically to measure the chemiluminescence and electrochemical transducer principles that will be employed, thus eliminating the need for bulky and expensive equipment like scanners (which are needed in systems like DNA micro-arrays). The light output will be measured using a photodiode or an active pixel array sensor. Electrical contacts (aluminum) patterned along the edges of the patterned NTS platform will be used for electrical measurements (cyclic voltammetry). Using appropriate data acquisition, a computer interface will be provided using either a serial or USB port communication.

3.2 Potential Difficulties

Going into the research, some difficulties were anticipated. Consistency in fabrication of NTS with uniform size and thickness characteristics could be difficult when using the anodization etching technique. This would introduce a large variation in the measured output of the biosensor from repeated experiments.

Direct binding (immobilization) of enzymes, antibodies, and DNA probe to the NTS surfaces could provide possible challenges; therefore, two procedures were investigated. Other silanizing agents (e.g., 3-aminopropyltriethoxy silane) and cross-linking (e.g., glutaraldehyde, avidin-biotin) could be used if 3-glycidoxypropyltrimethoxy silane and diffusion/physical adsorption proved to be ineffective for binding the biological sensing elements to the silicon surface.

3.3 Expected Significance

The expected outputs were the construction and validation of a "proof-of-concept" prototype NTS biosensor for rapid detection of *E. coli* and *Salmonella* that combined the current techniques in nanotechnology and biotechnology. This research would ultimately provide a highly self-contained, nucleic acid-based, specific, and sensitive, handheld

nanoscale-biosensor that has multiple, target-specific, electronically addressable, spots for on-site diagnosis of various pathogens (e.g., bedside diagnosis and point-of-care by health professionals). The prototype biosensor would be an added tool to protect the public from foodborne illness caused by contaminated food and water supply, reduce the health risk of microbial contamination, strengthen biosecurity measures, and enable quality assurance in food industry. The biosensor performance criteria included: (1) high sensitivity; (2) high specificity; (3) field-ready testing capacity; (4) simplicity ; and (5) short time requirements. The biosensor may be used for epidemiology, i.e., quick and effective diagnosis of disease outbreaks caused by pathogens either intentionally (bioterrorism) or inadvertently (lack of sanitation) added to air, food, and water. The biosensor could be easily adapted to several pathogens of concern, such as *Escherichia coli* O157:H7, *Listeria*, and *B. anthracis* by changing the specificity of the nucleic acid primers and probes used.

The current research developed the NTS biosensor platform, a versatile platform that was used for detection of three different targets with a high level of sensitivity (for example, pg level for DNA-based detection) and specificity (no cross reaction with related and non-related species), as well as detection time of 60 min or less. Two different kinds of transduction mechanisms and three biological sensing elements were employed successfully to develop the biosensors.

3.4 HYPOTHESIS

The research was based on the following hypothesis:

- A versatile nano-tubular platform can be fabricated using silicon and functionalized into a biosensor to detect foodborne pathogens using different biological sensing elements, such as enzymes, antibodies, and nucleic acids.

- The NTS platform can be used with multiple transducing mechanisms to report the biological detection event.
- The biological detection event on the NTS biosensor will trigger a response that is unique from that of a 'no detection' event, and which can be processed and converted into an appropriate electronic signal output.
- The NTS platform will provide greater sensitivity or lower limit of detection as compared to any planar platform of the same dimension (length and width).

3.5 SPECIFIC RESEARCH OBJECTIVES

Objective 1: Development of a chemiluminescence-based detection assay external to the biosensor system.

Objective 2: Design, fabrication and characterization of the NTS platform.

Objective 3: Functionalization of the NTS platform into a biosensor chip.

Objective 4: Determination of sensitivity of the NTS biosensor chip in pure culture.

Objective 5: Determination of specificity of the NTS biosensor chip using non-specific and mixed bacterial cultures.

CHAPTER 4: RESEARCH MATERIALS AND METHODS

4.1 OBJECTIVE 1

Development of a chemiluminescence-based detection assay external to the biosensor system to detect *E. coli* using β-galactosidase as the biological sensing element.

This objective was aimed at developing and optimizing a chemiluminescence-based assay protocol, external to the biosensor system, prior to developing the biosensor in Objective 2. Development of this assay protocol would then limit confounding variables during biosensor development to those related to the biosensor fabrication and functionalization.

4.1.1 Instrumentation

The chemiluminescence assay works on the basis of photon emission resulting from a 4-step reaction between the β-galactosidase enzyme produced by *E. coli* and the dioxetane in the Lumi-Gal® formulation. Total light emitted from the reaction is measured in a commercial luminometer (Femtomaster FB 12 Luminometer by Zylux Corp., Maryville, TN) equipped with a single photon-counter having a special measuring geometry that enables very high sensitivity (Figure 4-1). The sample is placed in a pullout sample holder that slides over a photo-multiplier tube (PMT). The sample holder focuses the light down toward the PMT. The PMT operates in the digital pulse mode wherein it acts as a photon counter. The light signal is processed and the photon counts are integrated over a period of 10 s. The digital readout is registered on the instrument; it can also be stored in a computer by using appropriate data acquisition software/hardware provided by the luminometer supplier.

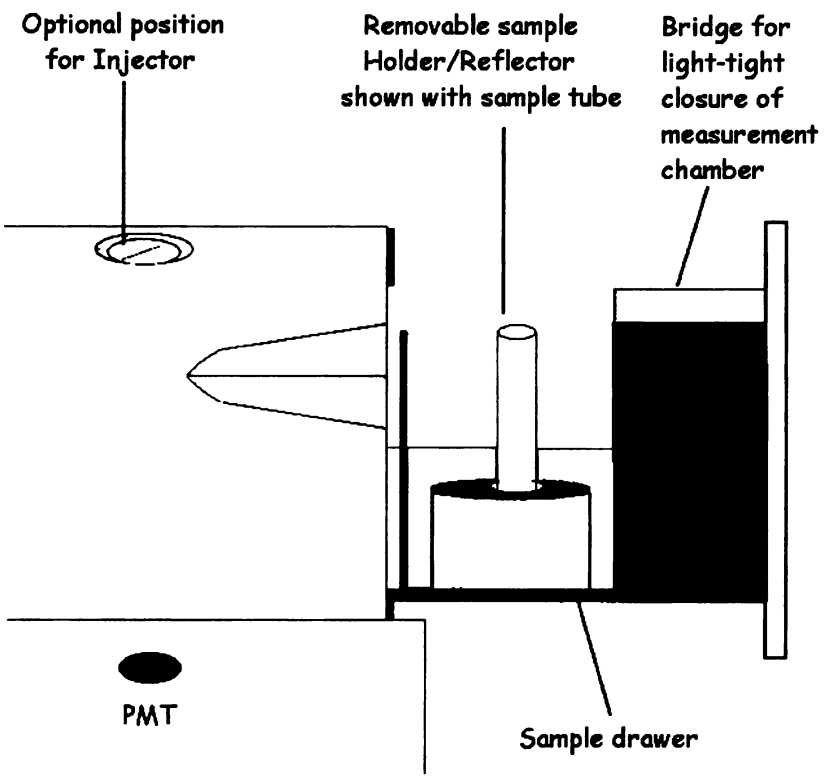

Figure 4-1. Schematic of the Femtomaster FB 12 Luminometer

4.1.2 Culture Preparation

Generic *Escherichia coli* (strain K-12) and *E. coli* O157:H7 (strain AD317) were obtained from the Department of Food Science and Human Nutrition, Michigan State University. The cultures were maintained at -70°C in trypticase soy broth (Becton Dickinson and Co., Cockeysville, MD) containing 10% (v/v) glycerol (J. T. Baker, Phillipsburg, NJ) and subjected to two consecutive overnight transfers (18-24 h/35°C) in 9 ml of nutrient broth (NB) (Difco, Becton Dickinson and Co., Sparks, MD) or lactose broth (LB) (Difco).

4.1.3 Single Tube Chemiluminescence Assay Procedure

Polymixin B sulfate (50mg/ml) solution (Sigma Chemical Co., St. Louis, MO) was added (as the *E. coli* cell wall permeabilizer) at a rate of 5 µl/ml culture to 9 ml of the

above *E. coli* culture or uninoculated NB (used as control) prior to incubation at 37°C for 15 min. From this mixture, 25 µl was then pipetted into a 10x75mm culture tube. After the addition of 500 µl Lumi-Gal® 530 (Lumigen, Inc., Southfield, MI), the tube was vortexed. The emitted light intensity was measured in relative light units per second (RLU/s) using the luminometer at 10 min intervals up to 60 min, and again at 75 and 90 min. Also, the number of *E. coli* was determined by spread plating the appropriately diluted sample on MacConkey agar (Difco). Bacterial counts were enumerated after 48 h of incubation at 35°C.

4.1.4 Determining Best Growth Media

Escherichia coli cultures were grown overnight in LB as well as NB and assay procedure repeated as above in both cases. A comparison of the results between NB and LB was used to determine which broth was better for the assay in terms of higher light output. A significantly higher light output from a sample containing low number of target cells over the control sample would improve biosensor sensitivity. Also, a comparison of light emitted after 24 h and 48 h of incubation of *E. coli* cultures was made to investigate the effect of incubation period on light intensity.

4.1.5 Determining Assay Applications

The above assay was also performed with *E. coli* O157:H7 cultures to determine possible differences in light emission when compared to the generic *E. coli* strain resulting from different levels of β-galactosidase enzyme production and/or activity. Such differences could be used as a discriminatory test between *E. coli* O157:H7 and generic *E. coli*. Also, overnight cultures of *E. coli* O157:H7 were heat treated prior to

testing with the chemiluminescence assay to determine if whether the assay could be employed for sterility testing with heat-processed foods, such as pasteurized milk.

4.1.6 Determining Amount of Sample to Use in the Assay

Following standardization of the assay procedure, a Randomized Complete Block Design (RBD) analysis, with 5 blocks of size 21 comprising a two-way treatment structure with 7 dilution and 3 *E. coli* culture levels, was used to assess the sensitivity of the assay. The randomized trials were performed for 5 consecutive days [determined by Power analysis of some preliminary data, performed using Statistical Analytical System (SAS© Version 8.0, SAS Institute, Inc., Cary, NC)] to nullify the effect of minor day-to-day variation in bacterial counts. *Escherichia coli* culture was serially diluted 5 times using 9 ml of 0.1% peptone dilution blanks, followed by the addition of 45 µl of polymixin B sulfate. From each dilution, 25 µl, 50 µl, and 55 µl were pipetted separately into 10x75mm culture tubes, and the assay procedure described above was followed thereafter. Peptone blank was used as the control in each case.

4.1.7 Assay Validation in Food Analysis

The chemiluminescence assay, developed and optimized using *E. coli* cultures in broth, was employed to detect *E. coli* in artificially contaminated food samples (pasteurized milk, sprouts, and water) so as to simulate a potential real-life usage scenario. Nine ml of water and pasteurized milk samples inoculated with different *E. coli* concentrations using overnight LB cultures were tested without any enrichment using the assay procedure. One gram of alfalfa sprouts inoculated with *E. coli* using the procedure of Beuchat *et al.* (2001) was added to 9 ml of sterile 0.1% peptone dilution blank. The mixture was hand mixed for 1 min. Five ml of liquid was removed and used for the assay

procedure as described above. All samples were tested in triplicate. Results of light emission from the samples were compared to coliform counts obtained from the MacConkey Agar plates for all samples (as described in the assay procedure).

For assay validation, blind sample testing was performed using surface water samples collected at seven sites along the Red Cedar River (East Lansing, MI). The water samples were contained in sterile 100 ml sampling bottles and transported to the lab facility. Nine ml was removed from the samples (without any enrichment) and analyzed using the assay procedure. One ml of the water sample was also transferred into each of a series of tubes containing 9 ml of LB and incubated at 35°C for a period from 1 to 6 h (hourly intervals) with an additional tube incubated for 12 h. The assay was repeated following each enrichment period for all samples.

4.1.8 Statistical Analysis

Analysis of Variance (ANOVA) was performed on the data using the SAS software. Arithmetic means were compared using the Duncan grouping test at 95% confidence level (α=0.05). Interactive effects in RBD were analyzed using the Autoregressive Mixed Model (Proc Mixed) with Tukey adjustment for comparison of means (α=0.05).

4.2 Objective 2

Design, fabrication and characterization of a nano-tubular silicon platform.

The prototype biosensor (in this research) was designed keeping in mind the long-term goal of building a self-contained nucleic acid-based individually addressable portable biosensor. In the current research, the process of PCR for DNA amplification was performed externally. The amplified product was then introduced to the prototype biosensor shown (Figure 4-2). The biosensor was fabricated on a p^{++}-type silicon 4"

wafer with final chip dimensions of 1.5 cm x 1.5 cm. The main fabrication steps are described below.

4.2.1 Nano-Tubular Silicon Fabrication

NTS was fabricated using the anodization process in a conventional single-tank cell (Figure 4-3). The O-ring was placed on the bottom hole of the inverted Teflon cell. The silicon wafer was then placed over the O-ring. Before the copper plate was placed over the silicon, the back surface of the silicon was scratched with a diamond-tipped scribe followed by application of graphite coating using a graphite pen to make the silicon more conductive. The copper metal contact was then made to the backside of the wafer and sealed. A platinum grid or wire placed inside the Teflon cell served as the cathode, and the copper plate served as the anode. The platinum grid (or wire) and the copper plate were connected to the negative and positive terminal of the power supply, respectively. After placing the Teflon cell upright, 15% hydrofluoric acid (in ethanol) was poured into the cell. Silicon was then electrochemically etched in the 15% hydrofluoric acid (HF) solution using different current and time conditions as follows: a) 100 mA – 30 s, b) 50 mA – 1 min, c) 30 mA – 1 min, d) 15 mA – 1 min, e) 5 mA – 20 min, f) 5 mA – 1 h, and g) 2 mA – 1 h. These current-time conditions were chosen by modifying those used for porous silicon fabrication (Halimaoui, 1997) so as to obtain the desired NTS platform.

4.2.2 Characterization of Nano-Tubular Silicon

SEM images of all NTS chips were taken using a Hitachi S-4700 II Field Emission Scanning Electron Microscope to evaluate the quality of nano-porous silicon chips obtained (detailed procedure provided in Appendix A:Section A.2). Images were

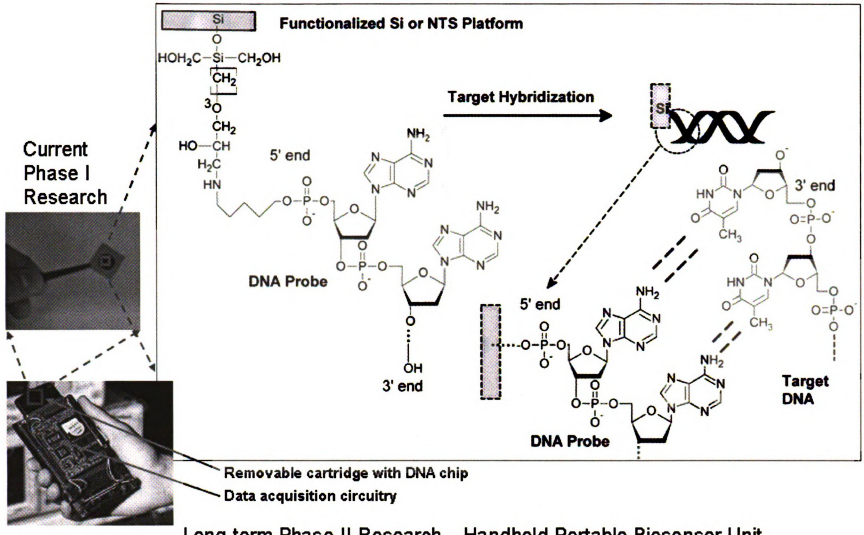

Figure 4-2. Biosensor schematic showing envisioned long-term and short-term research biosensors.

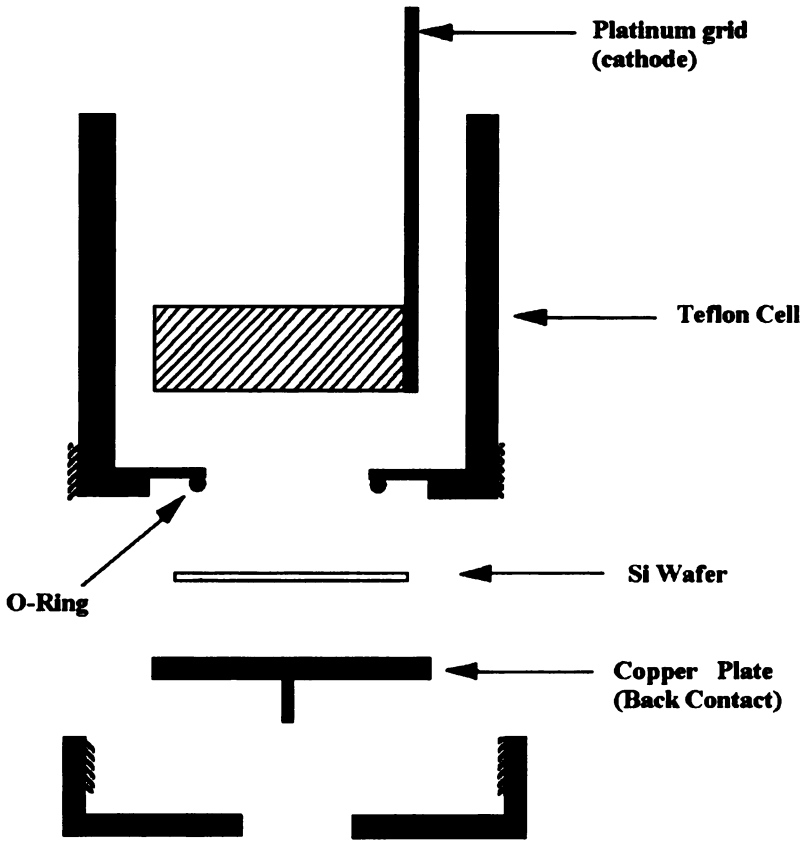

Figure 4-3. Single-tank cell set up used for tubular silicon fabrication.

used to observe the size and shape of the tubes, inter-tube space, surface texture, and thickness of the nano-tubular layer obtained under different anodization conditions described above.

The porosity and thickness of the porous silicon layers were also determined. The porosity was determined by weight measurements. The virgin wafer chip was first weighed before anodization (m_1), then just after anodization etching (m_2) and finally after complete dissolution of the NTS layer in NaOH solution (m_3). The final dissolution process was carried out in the Teflon cell used for anodization. The chip, after weighing (m_2), was carefully placed under the cell (as described above in Section 4.2.1) and exposed to an aqueous solution of 2.5 M NaOH for a period ranging between 30-90 min

(Vázsonyi *et al.*, 2003) until the shiny clear surface of planar silicon was visible again. The chip was then weighed again (m_3).

The porosity is given by the following equation:

$$P(\%) = \frac{(m_1 - m_2)}{(m_1 - m_3)} \times 100$$

From the above measurements, the thickness of the porous silicon layer was also determined as follows: $W = \frac{(m_1 - m_3)}{S \times d}$; where,

d = Bulk silicon density, (2.33 g/cm^3), and

S = Area of the wafer exposed to HF during anodization (circular area of 1.1 cm diameter = 0.95 cm^2).

4.3 OBJECTIVE 3

Functionalization of the nano-tubular silicon platform into a biosensor chip.

4.3.1 Adaptation of CL Assay to the Biosensor Detection System - Procedure A

4.3.1.1 Functionalization of nano-tubular silicon with Lumi-Gal® 530

After fabrication of NTS, the platform was cleaned by rinsing with isopropyl alcohol and then dry toluene, followed by drying under a steady stream of nitrogen for 30 s. To functionalize the NTS platform, 50 µl of Lumi-Gal® 530 was carefully added to the NTS surface, which was then placed overnight in the refrigerator (4°C) to allow binding of Lumi-Gal® 530 by diffusion and adsorption. The biosensor chip was then dried by placing it in a vacuum oven (Isotemp vacuum oven 280A, Fisher Scientific, Pittsburg, PA) at 37°C for 1-2 h (25 in Hg). The biosensor chip was then ready to be used for detection of *E. coli* using β-galactosidase enzyme as the marker. Planar Si chips were

also functionalized using the above process and used to compare and evaluate the effectiveness of the NTS biosensor chip as a biosensor platform.

4.3.1.2 Detection of E. coli *on biosensor chip using CL detection*

Polymixin B sulfate (50 mg/ml) solution (Sigma Chemical Co., St. Louis, MO) was added (as the *E. coli* cell wall permeabilizer) at a rate of 5 µl/ml culture to 9 ml of the overnight *E. coli* culture (Section 4.1.2 above) or uninoculated LB (used as control) prior to incubation at 37°C for 15 min. From this mixture, 5 µl was then added carefully to the biosensor chip. The biosensor was inverted before placing in the luminometer because the photomultiplier tube detects the light from below the sample drawer. The emitted light intensity was measured in relative light units per second (RLU/s) using the luminometer at 10 min intervals up to 60 min. Light emission of the functionalized NTS biosensor chip was also compared with that of a functionalized planar Si chip to evaluate the performance of the non-planar biosensor platform being developed. The light emission data collected at the third 10-min interval, established as ideal based on prior results (Mathew and Alocilja, 2002), were used for the comparisons. The trials were performed in triplicate.

4.3.2 Adaptation of CL Assay to the Biosensor Detection System - Procedure B

4.3.2.1 Single-tube CL assay modification

In order to adapt the single-tube assay external to the biosensor (Section 4.1.1-4.1.6) (Mathew and Alocilja, 2004) to the NTS biosensor, the CL assay steps were modified as follows: polymyxin B sulfate was pre-mixed with dioxetane by adding 5 µl of polymyxin B sulfate (50 mg/ml) solution per ml of the dioxetane substrate. From this polymyxin B-dioxetane mixture, 500 µl was pipetted into a 10x75 mm culture tube followed by

addition of 55 µl of the *E. coli* culture (Section 4.1.2); the tube was then vortexed. The emitted light intensity was measured using the luminometer at 10 min intervals up to 60 min and compared with that of the single-tube assay procedure described above. These modifications were aimed at minimizing the assay steps and the assay time required to obtain an output from the biosensor.

4.3.2.2 Functionalization of nano-tubular Si

After fabrication of the NTS chip, it was cleaned by rinsing with isopropyl alcohol and then dry toluene, followed by drying under a steady stream of nitrogen. To functionalize the NTS platform, 50 µl of the polymyxin B-dioxetane mixture (as described above) was carefully added to the porous silicon surface and placed overnight in the refrigerator (4°C) to allow binding of the mixture on the surface through diffusion and adsorption. The biosensor chip was then partially dried by placing it in a vacuum oven (Isotemp vacuum oven 280A, Fisher Scientific, Pittsburg, PA) at 37°C for 1-2 h (25 in Hg). At this stage, the biosensor chip was ready for use.

Planar silicon chips were also functionalized using the above process and used to compare and evaluate the effectiveness of the porous silicon biosensor chip as a biosensor platform.

4.3.2.3 Biosensor testing

Five microliters of the overnight *E. coli* culture or uninoculated LB (used as control) was added carefully to the functionalized biosensor chip. The chip was placed in the luminometer, and the emitted light was measured in RLU/s at 10 min intervals up to 60 min. Light emission of the functionalized NTS biosensor chip was also compared with that of a functionalized planar silicon chip to evaluate the performance of the NTS

biosensor platform being developed. The light emission data collected at the third 10-min interval, established as ideal based on prior results (Mathew and Alocilja, 2004), were used for comparison. The trials were performed in triplicate.

4.3.2.4 Determining the biosensor sensitivity

Following standardization of the assay procedure for the biosensor, a completely randomized design (CRD) was used with 5 levels of dilution (including blank/control) to assess the sensitivity of the assay. The randomized trials were performed in triplicate to nullify the effect of minor day-to-day variation in bacterial counts. *Escherichia coli* culture was serially diluted 5 times using 9 ml of 0.1% peptone dilution blanks. From each dilution (10^1-10^4 cells), 5 µl were pipetted separately on to functionalized biosensor chips, and the modified assay procedure described above was followed thereafter. Peptone blank was used as the control.

Statistical Analysis

F-test was used to test equality of variances. Two-sample t-test was used to test the mean differences between the light emission observed using the standard and modified chemiluminescence assay procedures. Single-factor Analysis of Variance (ANOVA) was performed on the light emission data for the sensitivity analysis. Arithmetic means were compared using Duncan grouping at 95% confidence level (α=0.05). The performance of the biosensor using Procedures A and B were compared to determine possible significant differences in sensitivity. All statistical analyses were carried out using SAS® software (SAS, Gary, NC).

4.3.3 Functionalization of the Nano-Tubular Silicon Platform with *Salmonella* Antibodies into an Immunosensor

4.3.3.1 Silanization of nano-tubular silicon platform

The NTS surface was functionalized by silanization and antibody immobilization based on a procedure described previously by Bhatia *et al.* (1989) (Figure 4-4). NTS was first cleaned by soaking in a 50/50 solution of methanol and hydrochloric acid. After rinsing with deionized water several times, NTS was treated with concentrated sulfuric acid for 30 min to hydrate the silicon surface. The chips were rinsed again with deionized water and then air dried. Following cleaning, NTS was silanized with a 2% solution of 3-glycidoxypropyltrimethoxysilane (GOPS) (Aldrich Chemicals, Allentown, PA) in dry toluene for2 h. Silanization was done in a glove box under inert atmosphere. The NTS substrate was then rinsed in dry toluene and allowed to air dry.

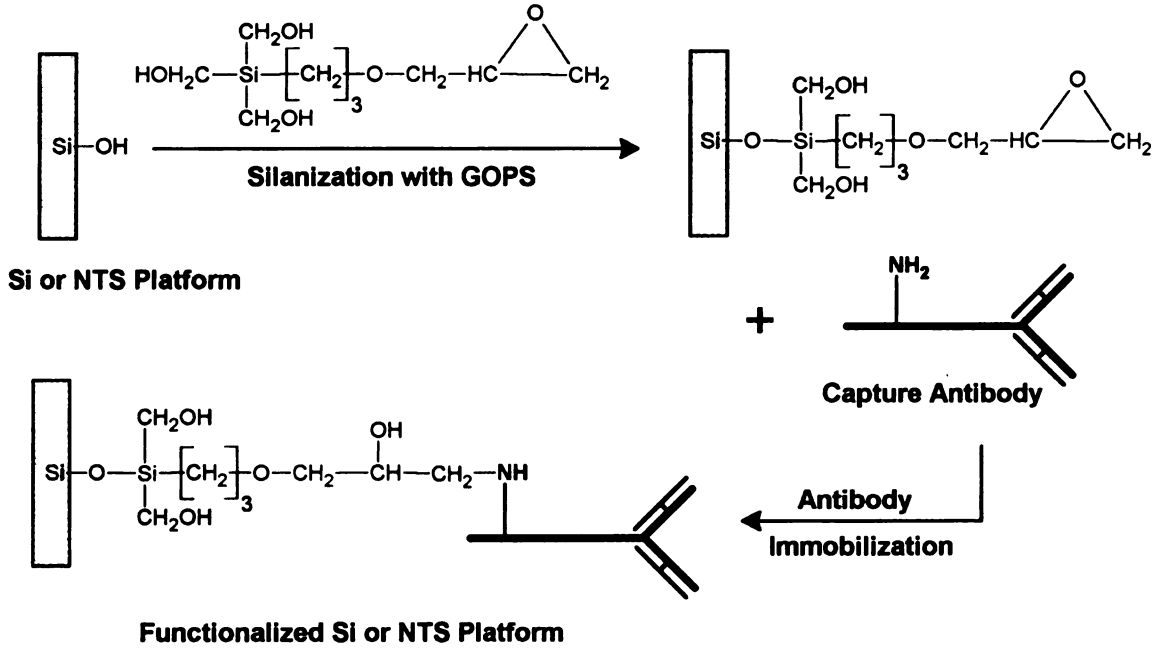

Figure 4-4. Functionalization of planar Si and nano-tubular Si platforms into antibody-based biosensors.

4.3.3.2 Immobilization of primary antibody

Following silanization, 0.5 ml of a 0.15-mg/ml solution of anti-*Salmonella* antibody, targeting the common structural antigen of *Salmonella* (KPL, Gaithersburg, MD), was carefully pipetted on to the active NTS area and then allowed to attach to the silanized NTS for 1 h. NTS was rinsed with phosphate buffered saline (PBS, pH 7.4) and stored at 4°C until used. In order to study the influence of silanization on the functionalization of the biosensor, half of the NTS chips were subjected to all the above steps except for the 2 h silanization.

4.3.3.3 Optical measurement method

A CL-based optical method was used to detect the presence of *Salmonella* on the biosensor chip surface. A half milliliter of overnight *Salmonella* Typhimurium DT104 (from Michigan State University collection) culture grown in lactose broth was added to the nano-tubular- and planar-silanized chips as well as the non-silanized NTS biosensor chips. A half milliliter of sterile buffer was added to the control chips. After five minutes, each biosensor chip was rinsed with 1 ml of 0.1% peptone dilution blank. Half milliliter of blocking solution (2% albumin in PBS) was then added. After 10 minutes, the blocking solution was drained. A 1:20000 solution of the secondary anti-*Salmonella* antibody conjugated with horseradish peroxidase (HRP) (KPL) was then added to cover the active porous silicon area (approximately 0.5 ml). After 15 minutes, the biosensor was rinsed 3-4 times with 1 ml each of PBS containing 0.1 % Tween-20 solution to remove unattached HRP-conjugate. One ml of Luminol® (Pierce Biotechnology, Rockford, IL) was pipetted into a 35 mm petri dish and then the biosensor was inverted into the petri dish. Chemiluminescence from the reaction between Luminol® and HRP was measured in the luminometer at 1 min intervals for 10 min. Bacterial numbers were

obtained by plating appropriately diluted samples on trypticase soy agar (Difco, Becton Dickinson and Co., Sparks, MD) containing 0.6% yeast extract (Difco). The trials were performed in triplicate.

4.3.4 Functionalization of the Nano-Tubular Silicon Platform with *Salmonella* DNA Probes into a Nucleic Acid-Based Biosensor

4.3.4.1 Bacterial strains

A clinical strain of *Salmonella* Enteritidis (strain S-64) was obtained from the Michigan Department of Community Health (Lansing, MI) and stored at –70°C. The pathogen was grown on trypticase soy agar containing 0.6% yeast extract (TSAYE) and/or broth (TSBYE) at 37°C, as appropriate. In broth culture, cells were grown to exponential phase, and enumerated by spiral plating appropriately diluted cultures on Bismuth Sulfite Agar and Brilliant Green Agar. The cultures were serially diluted for DNA extraction so that the number of bacterial cells ranged from 10^0 to 10^8 CFU/ml.

4.3.4.2 DNA primers and probes

Primers used for PCR were designed for the detection of *Salmonella* Enteritidis from the insertion element (*Iel*) gene (Wang and Yeh, 2002). The single stranded forward and reverse primers were *Iel*L - 5'- CTAACAGGCGCATACGATCTGACA -3' (positions 542 to 565, 24 bases) and *Iel*R – 5'- TACGCATAGCGATCTCCTTCGTTG -3' (positions 1047 to 1024, 24 bases). Capture probe used was 5'- [Amino link] AATATGCTGCCTACTGCCCTACGCTT -3' (positions 690 to 716 of target, 26 bases).

4.3.4.3 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed with Taq DNA polymerase in a DNA thermal cycler. The 50 µl PCR reaction mix consisted of 0.5 µM of the above primers set, 25 µl of Promega Master Mix (Promega Corporation, Madison, WI), containing the

proprietary reaction buffer (pH 8.5), 200 μM deoxynucleotide triphosphates (dNTP), 3 mM $MgCl_2$, and 2.5 U of Taq DNA polymerase. A *Salmonella* colony , grown overnight on TSAYE, was suspended in 0.5 ml of DNA grade water (Fisher Scientific, Pittsburg, PA) containing 5 mM of NaOH, and boiled for 10 min to rupture the cells. Alternately, 0.1 ml of an overnight *Salmonella* culture grown in TSBYE was mixed with 0.4 ml of distilled DNA grade water containing 5 mM of NaOH and boiled for 10 min. As a template, 1 μl was added to the PCR reaction mixture. The cycling reaction was performed as follows: heat denature at 94°C for 5 min with 30 additional cycles of heat denature at 94°C for 30 s, primer annealing at 59°C for 30 s, and extension at 72°C for 45 s. After the final cycle, the samples were maintained at 72°C for 10 min to complete DNA strand synthesis and then cooled to 4°C, unless used immediately.

4.3.4.4 Silanization of nano-tubular Si platform

Figure 4-5 illustrates the functionalization of the NTS platform into a DNA biosensor, comprising two main steps – silanization and DNA oligonucleotide probe immobilization. For silanization, the NTS substrates were first cleaned in boiling acetone for 5 min, boiling methanol for 5 min, and then dried under nitrogen. The substrates were immersed in sulfochromic acid (strong oxidizing agent) for 15 min to hydrate and prepare the surface for silanization. Following the acid treatment, the substrates were washed twice in deionized water and dried under nitrogen. The substrates were then placed in a clean oven for 1 h at 140 °C. Immediately after heating, the substrates were transferred to a glove box under a dry nitrogen atmosphere. A 10% 3-glycidoxypropyltrimethoxysilane (GOPS) solution (in dry toluene) was placed on the substrates. The substrates were left to react under nitrogen for 4 h, then rinsed with toluene and left to dry. A background

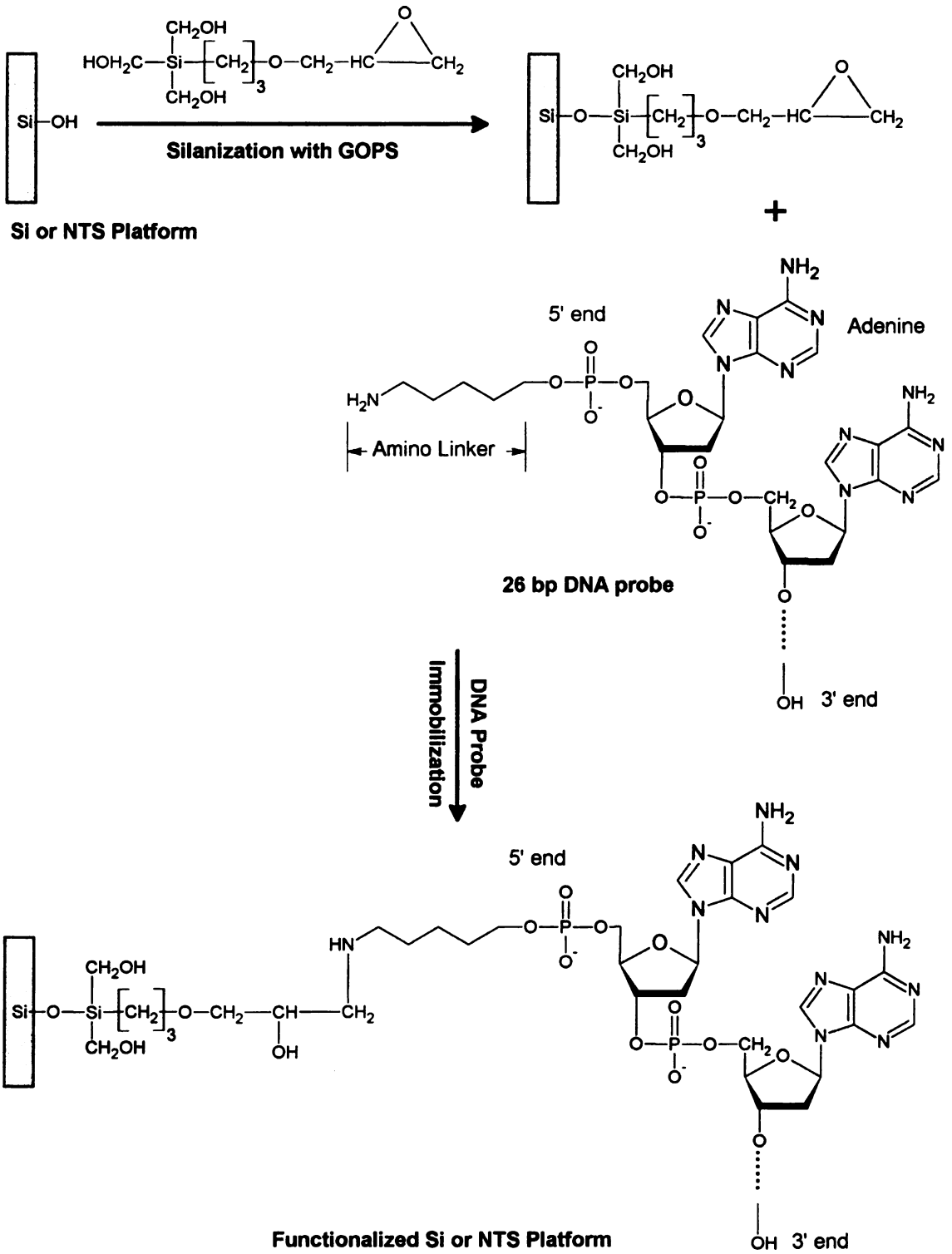

Figure 4-5. Functionalization of planar Si and nano-tubular Si platform into DNA-based biosensor.

electrochemical cyclic voltammetry (CV) reading was then taken prior to DNA probe immobilization.

4.3.4.5 DNA probe immobilization

Immobilization of the probe layer was performed by coating each substrate with 50 µl of 150 nM aqueous DNA probe solution (in 1 mM NaOH) specific to the pathogen of interest, and leaving them to react overnight at room temperature in the glove box. The substrates were finally placed in boiling water for 2 min to remove excess unreacted DNA probe and dried under nitrogen. The unreacted aldehyde groups of GOPS were then saturated by dipping the chips in a 0.1 M glycine solution for 20 min. Chips were washed using a washing solution (1X SSC containing NaCl and Na-citrate – see Appendix B:Section B.2 for more details) and dried under nitrogen. Electrochemical cyclic voltammetry (CV) reading was taken prior to target DNA hybridization. The prototype biosensor was stored under nitrogen at 25 °C for maximum stability, until used.

4.3.4.6 Characterization of the DNA

Following PCR, the size of the PCR amplified DNA product was determined using gel electrophoresis (procedure in Appendix A:Section A.3). The DNA concentration was also determined using Bio-Rad SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA) readings at 260 and 280 nm. PCR amplified DNA was diluted to 10^{-9} µg/µl in hybridization buffer (5X SSC, 0.1% SDS, 0.5% BSA) and used for initial characterization of the NTS DNA-based biosensor.

4.3.4.7 Target DNA hybridization

DNA in the hybridization buffer was denatured in a 95°C water bath, and cooled to 59°C (just below melting temperature of the capture probe). The hybridization of complementary DNA strands (obtained by extracting DNA from the sample of interest)

with the immobilized oligonucleotide probe layer was performed by dipping the DNA modified chip in a solution of the target DNA at the appropriate concentration in hybridization buffer for 45-60 min at 59°C (Figure 4-6). The non-specifically adsorbed strands were then removed by extensive washing in Wash solution A (0.1X SSC, 0.1% SDS – see Appendix for more details) followed by Wash solution B (0.1X SSC – see Appendix for more details). The chips were then dried under nitrogen and CV readings measured. Optimum operating conditions for the biosensor were determined.

4.3.4.8 Electrochemical detection - cyclic voltammetry

Before adding the target DNA, a baseline was obtained for each sensor (after DNA probe immobilization) using a blank containing an aqueous solution of 5 mM potassium ferrocyanide in 1 M potassium nitrate. A volume of 25 ml was added to a three-electrode electrochemical cell (Figure 4-7). Cyclic voltammetry was performed on the blank solution using a Versastat II Potentiostat/Galvanostat (Princeton Applied Research, Oak Ridge, TN). The working electrode was the treated biosensor platform, the reference electrode was the Ag/AgCl electrode, and the counter electrode was a graphite electrode rod. The potentiostat was run in the ramp, one vertex multi mode. The potential was cycled between –1.2 V to +1.5 V at a scan rate of 20 mV/s. The resulting current was measured and plotted against the potential. After measurement, the blank solution was discarded. The procedure was repeated with the biosensor following target DNA hybridization.

4.3.4.9 Confirmation of results

Atomic Force Microscopy images were used to determine surface level changes occurring due to immobilization of the DNA probe and the target DNA hybridization.

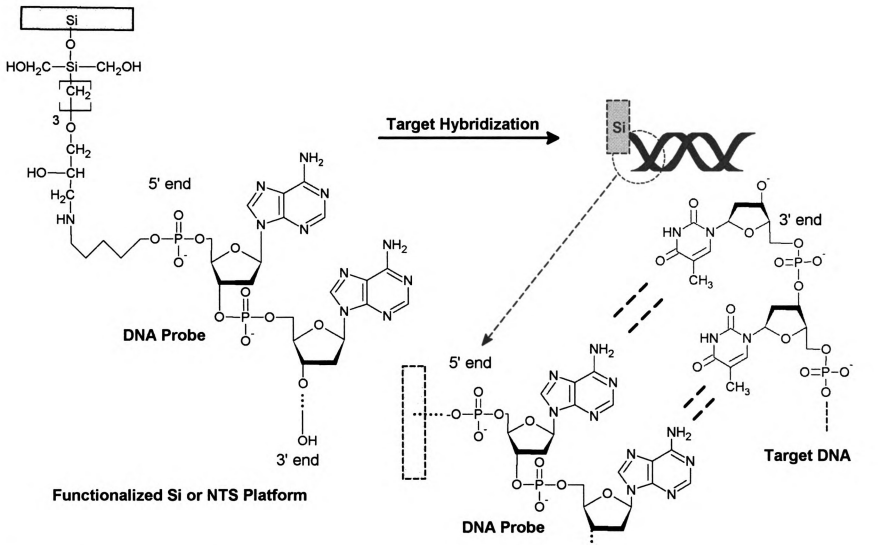

Figure 4-6. Schematic of target DNA hybridization to immobilized DNA probe on functionalized biosensor surface.

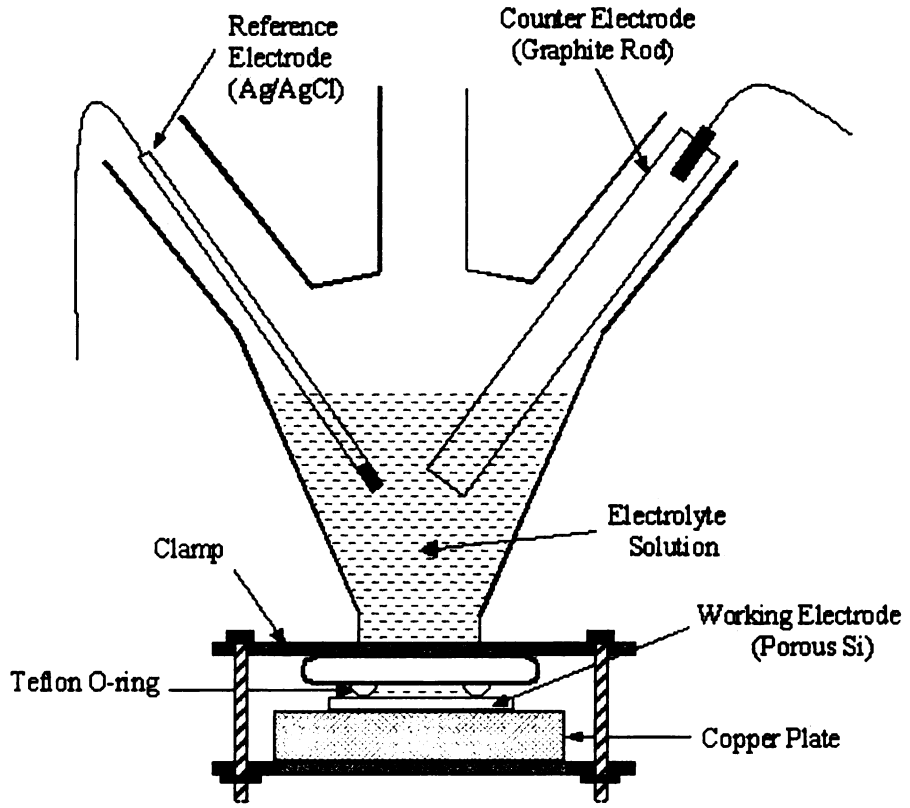

Figure 4-7. Three-electrode electrochemical cell used for cyclic voltammetry measurements.

4.3.4.10 Statistical analysis

For each set of experiments, the raw data consisted of plots of Current (I) vs. Potential (E) curves with data from two successive cycles (technical replicates) collected for analysis. Each cycle of the biological replicate consisted of 1020 pairs of points (E, I). Half of these points corresponded to the oxidation reaction and the other half corresponded to the reduction reaction of the cycle.

The maximum and minimum peak currents at the oxidation-reduction shifts on the cyclic voltammograms were selected for statistical analysis. Mean significant differences between the "peak current after addition of the DNA target" and "peak current before addition of the DNA target" were statistically analyzed to determine the presence or

absence of the target pathogen DNA in the sample. Such an approach uses only one single data point from the anodic and the cathodic side of the cyclic voltammogram. Alternate approaches used to analyze the data collected are described in Section 4.4.4.

4.4 OBJECTIVE 4

Determination of sensitivity of the nano-tubular silicon DNA biosensor in pure culture.

4.4.1 Sensitivity of the Biosensor Using PCR Amplified DNA

Overnight culture of *Salmonella* from Objective 3 (methods for part 3.4) was used to assess the sensitivity (lower limit of detection) of the biosensor. DNA was extracted and PCR performed. The PCR product was diluted serially so that the concentration ranged from 10^{-9} μg/μl to 10^{-15} μg/μl, followed by testing each DNA concentration with the NTS as well as planar Si biosensor as described in Objective 3 (Section 4.3.4.8) to determine the sensitivity of the biosensor.

4.4.2 Sensitivity of the Biosensor Using DNA from Pure Culture

An overnight *Salmonella* Enteritidis culture from Objective 3.4 was used to assess the sensitivity (lower detection level) of the NTS biosensor. DNA was extracted as described below, followed by serial dilution of the DNA from 10^{-9} μg/μl to 10^{-12} μg/μl (and lower, if necessary) for testing with the NTS biosensor and planar Si biosensor as described in the procedure for Objective 3 (Section 4.3.4.8). The sensitivity of the biosensor was then determined. This was done to evaluate the need for PCR of the DNA target prior to detection by the biosensor.

4.4.2.1 Isolation of genomic DNA from bacterial cultures

Genomic DNA was extracted from an overnight *Salmonella* Enteritidis culture using the QiaAmp DNA Mini Kit (Qiagen Inc., Valencia, CA). The procedure used was as follows:

1. One milliliter of the bacterial culture was pipetted into a 1.5 ml microcentrifuge tube. The tube was centrifuged for 5 min at 5000 x g (7500 rpm).
2. The volume of the pellet at the bottom of the centrifuge tube was estimated and Buffer ATL (tissue lysis buffer supplied in the QIAamp DNA Mini Kit) was added to a total volume of 180 µl.
3. 20 µl of Proteinase K was then added, tube content mixed by vortexing, and incubated at 56°C until the cells were completely lysed. The content was mixed by vortexing 2–3 times per h during incubation to disperse the sample. Lysis was complete in 3 h. The 1.5 ml microcentrifuge tube was briefly centrifuged to remove drops from the inside of the lid.
4. 200 µl of Buffer AL (lysis buffer supplied in the QIAamp DNA Mini Kit) was added to the sample, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min followed by brief centrifugation of the 1.5 ml microcentrifuge tube to remove drops from inside the lid.
5. 200 µl of ethanol (100%) was added to the sample, and mixed by pulse-vortexing for 15 s. The mixture in the tube from Step 4 was carefully applied (including any precipitate) to the QIAamp Spin Column (in a 2 ml collection tube, both provided in the QIAamp DNA Mini Kit) without wetting the rim, followed by centrifuging at 6000 x g (8000 rpm) for 1 min. The QIAamp Spin Column was then placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate discarded.

6. 500 µl Buffer AW1 (washing buffer supplied in the QIAamp DNA Mini Kit) was added to the QIAamp Spin Column without wetting the rim, and centrifuged at 6000 x g (8000 rpm) for 1 min. The QIAamp Spin Column was then placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate discarded.
7. 500 µl Buffer AW2 (washing buffer supplied in the QIAamp DNA Mini Kit) was added to the QIAamp Spin Column without wetting the rim, and centrifuged at full speed (10,000 x g; 14,000 rpm) for 3 min. The QIAamp Spin Column was then placed in a clean 2 ml collection tube (provided), and the tube containing the filtrate discarded.
8. 200 µl of Buffer AE (elution buffer supplied in the QIAamp DNA Mini Kit) was added to the QIAamp Spin Column and incubated at room temperature for 5 min, and then centrifuged at 6000 x g (8000 rpm) for 1 min.
9. Above step 8 was repeated once more to elute more DNA from the QIAamp Spin Column. For long-term storage, the eluted DNA was stored in Buffer AE at –20°C.

4.4.3 Confirmation of Results

Results from all of the above procedures were confirmed by following the standard plating method (Food and Drug Administration, 2000) and gel electrophoresis. DNA concentrations were determined using Bio-Rad SmartSpec 3000 spectrophotometer readings at 260 and 280 nm.

4.4.4 Statistical Analysis

All the above trials were performed in triplicate. For each set of experiments, the raw data consisted of plots of I vs. E curves with two technical replicates used for analysis. Each cycle of the biological replicate consisted of 1020 pairs of points (E, I). Half of

these points corresponded to the oxidation reaction and the other half corresponded to the reduction reaction of the cycle.

4.4.4.1 Analysis of curves (Modeling)

For each technical replicate cycle, oxidation and reduction reactions segments of the curve were analyzed separately. A generalized additive model was used to model the current as a function of voltage because this model has the flexibility of incorporating a semiparametric component (smoothing spline) to obtain a smoothed fit for two variables, especially when the functional form of the relationship is not known. PROC GAM of SAS was used to fit this model for each condition (combination of hybridization step, DNA concentration, DNA source, and DNA target). The two technical replications (cycle 1 and cycle 2) were averaged before fitting the generalized additive model. In addition to the smoothing curve, a 95 % confidence interval was calculated.

4.4.4.2 Analysis of Delta Q values

Another approach used to determine significant differences in the current signal output was to calculate the amount of charge (Q) passed during an experiment. Cummulative charge (ΔQ) values were obtained using the cyclic voltammetry *Powersuite* software (Princeton Applied Research) by selecting the whole set of 1020 points. The software calculated the ΔQ as the integral of current across the selected set of points with respect to time, and displayed it in a hoover box.

The ΔQ values (one for each cycle) were collected and analyzed using ANOVA models. Values from the two cycles were analyzed individually. In a first analysis, the three hybridization conditions were compared within each combination of concentration and DNA source (pure or PCR). A significant difference result from the ANOVA was

followed by a protected LSD (least significative difference) test. Subsequently, factorial ANOVA analyses (using PROC Mixed) were used to study the three-way interaction among hybridization, concentration and source as well as the double interaction of hybrydization with concentration. Depending on the results of these interaction tests, single or main effect contrasts among hybridization types were calculated. Finally, differences in performance between the two platforms, NTS and planar Si, were also determined.

4.5 OBJECTIVE 5

Determination of specificity of the nano-tubular silicon biosensor using mixed bacterial cultures.

4.5.1 Specificity Testing

In order to determine the specificity of the biosensor (the *Salmonella* DNA probes used), *Salmonella* cultures were mixed with related bacteria (*Escherichia coli*) at a 1:1 ratio. PCR was performed using these mixed as well as non-specific bacterial cultures (only *E. coli*) as described in Objective 3, Section 4.3.4. Genomic DNA was also extracted from an overnight *E. coli* K-12 culture. The extracted genomic *E. coli* DNA was then tested with the NTS biosensor alone (non-target) and in a mixture containing a 1:1 ratio of 1 ng/μl each of *S.* Enteritidis and *E. coli* DNA. The signal generated was compared statistically against negative and positive control samples of *Salmonella* Enteritidis DNA to determine the specificity of the biosensor.

4.5.2 Confirmation of Results

Results from all of the above procedures were confirmed by gel electrophoresis and spectrophotometer readings at 260 and 280 nm.

4.5.3 Statistical Analysis

All the above trials were done in triplicate. Analysis of the data was performed as described in Section 4.4 to determine significant differences in mean ΔQ values between and within species (*E. coli* or *Salmonella*). SAS program was used for statistical analysis.

CHAPTER 5: RESULTS AND DISCUSSION

The first step toward developing a versatile silicon-based platform for biosensor fabrication was to develop an assay and optimize its performance outside of the biosensor system so as to minimize the confounding variables. The assay was then used as a measure of comparison in terms of the biosensor performance vis-à-vis the assay performance external to the biosensor system. Upon successful fabrication of the biosensor platform and adaptation of the external assay to the biosensor, the versatility of the platform was demonstrated by employing two different transducer mechanisms with three biological sensing elements. The following sections will present the results obtained for the objectives outlined in the research methods (Chapter 4) aimed at developing the versatile silicon-based biosensor platform. A discussion of the obtained results in light of the current knowledge base is also provided.

5.1 OBJECTIVE 1

Development of a chemiluminescence-based detection assay external to the biosensor system to detect *E. coli* using β-galactosidase as the biological sensing element.

5.1.1 Development of Chemiluminescence-Based Assay

The chemiluminescence-based assay external to the biosensor system was successfully developed using β-galactosidase produced by *E. coli* as the marker. Generic *Escherichia coli* (K-12), grown overnight in nutrient broth (NB) to obtain cells in the late exponential or stationary phase, was used to develop the chemiluminescence-based assay. The kinetics of light emission from an overnight *E. coli* culture grown in NB is given in Figure 5-1. The mean *E. coli* population in the overnight culture was 8.36±0.11 Log CFU/ml (n=4). Light emitted from the uninoculated NB remained fairly steady over the

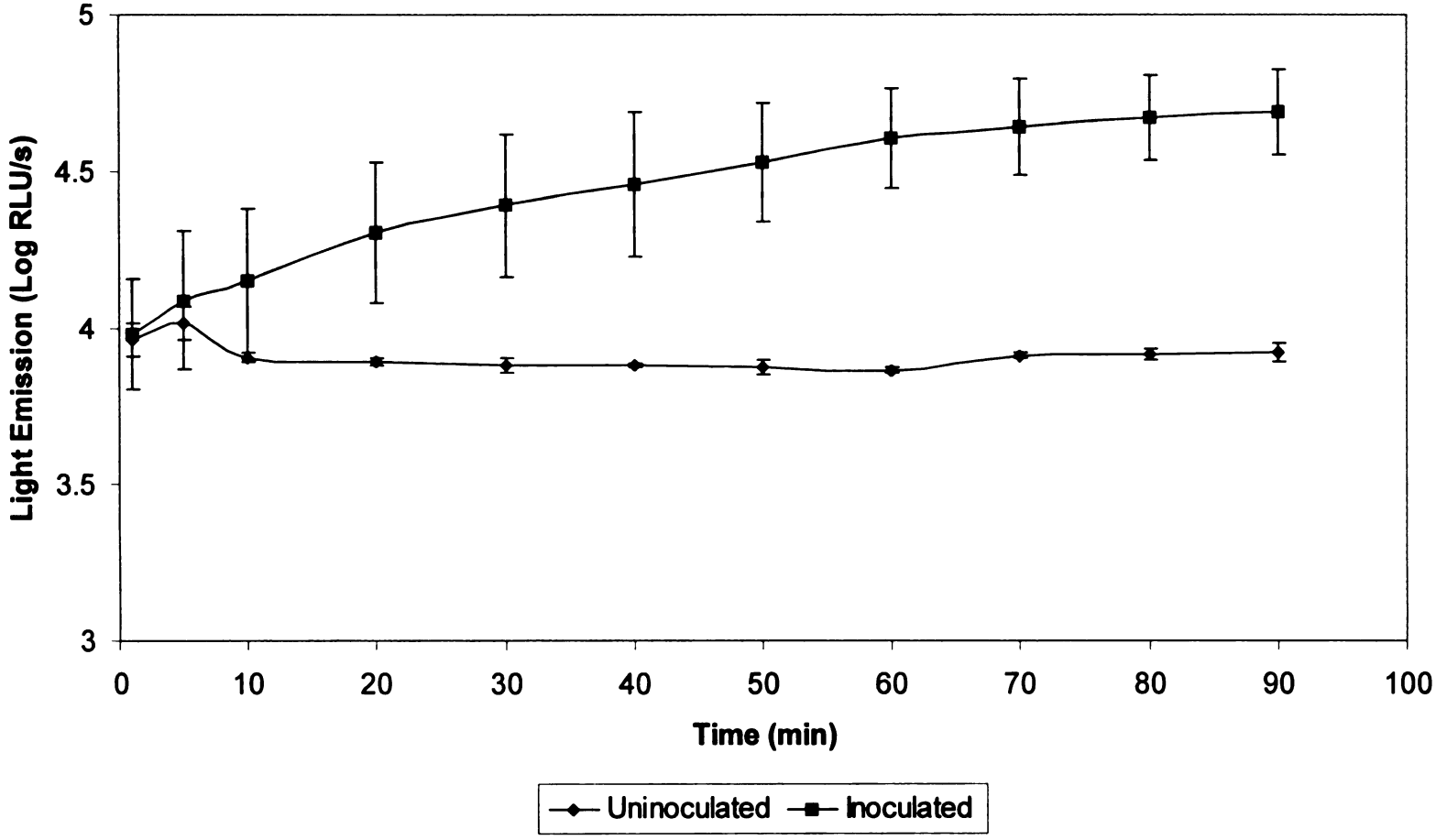

Figure 5-1. Kinetics of light emitted by an overnight NB *E. coli* culture.

entire period of light measurement (90 min) at 3.9-4.0 Log relative light units per second (Log RLU/s). Light intensity for the *E. coli* pure culture was similar to that obtained from the uninoculated control for the first 5 min, and then increased up to 60 min before approaching a plateau at 4.7 Log RLU/s. The light intensity remained almost steady at that level even after 6 h (data not shown). The difference in light emission between the uninoculated NB blank and the NB sample with an overnight *E. coli* culture was significant after 10 min of light measurement ($p<0.05$).

The β-galactosidase-based chemiluminescence assay for detection of *E. coli* was thus successfully demonstrated using NB as the growth medium. The kinetics of light emitted by an overnight NB *E. coli* culture was similar to that observed by Lumigen, Inc. (supplier of Lumi-Gal® 530) and other investigators (Beale *et al.*, 1992). Beale *et al.* (1992) observed a linear relationship between the *E. coli* β-galactosidase concentration and emitted chemiluminescence over five orders of magnitude.

5.1.2 Comparison Between Nutrient Broth and Lactose Broth

The effect of the growth medium in which the *E. coli* was cultured as well as the incubation period was investigated (Figure 5-2). A comparison of light emitted after 24 h and 48 h of incubation did not show any significant difference ($p>0.05$) when grown in NB or LB. Differences in *E. coli* numbers were also not significant (8.37 ± 0.10 Log CFU/ml and 8.23 ± 0.09 Log CFU/ml for NB after 24 and 48 h, respectively; versus 8.29 ± 0.08 Log CFU/ml and 8.20 ± 0.10 Log CFU/ml for LB after 24 and 48 h, respectively). However, the intensity of light emitted by *E. coli* cultures grown in NB and

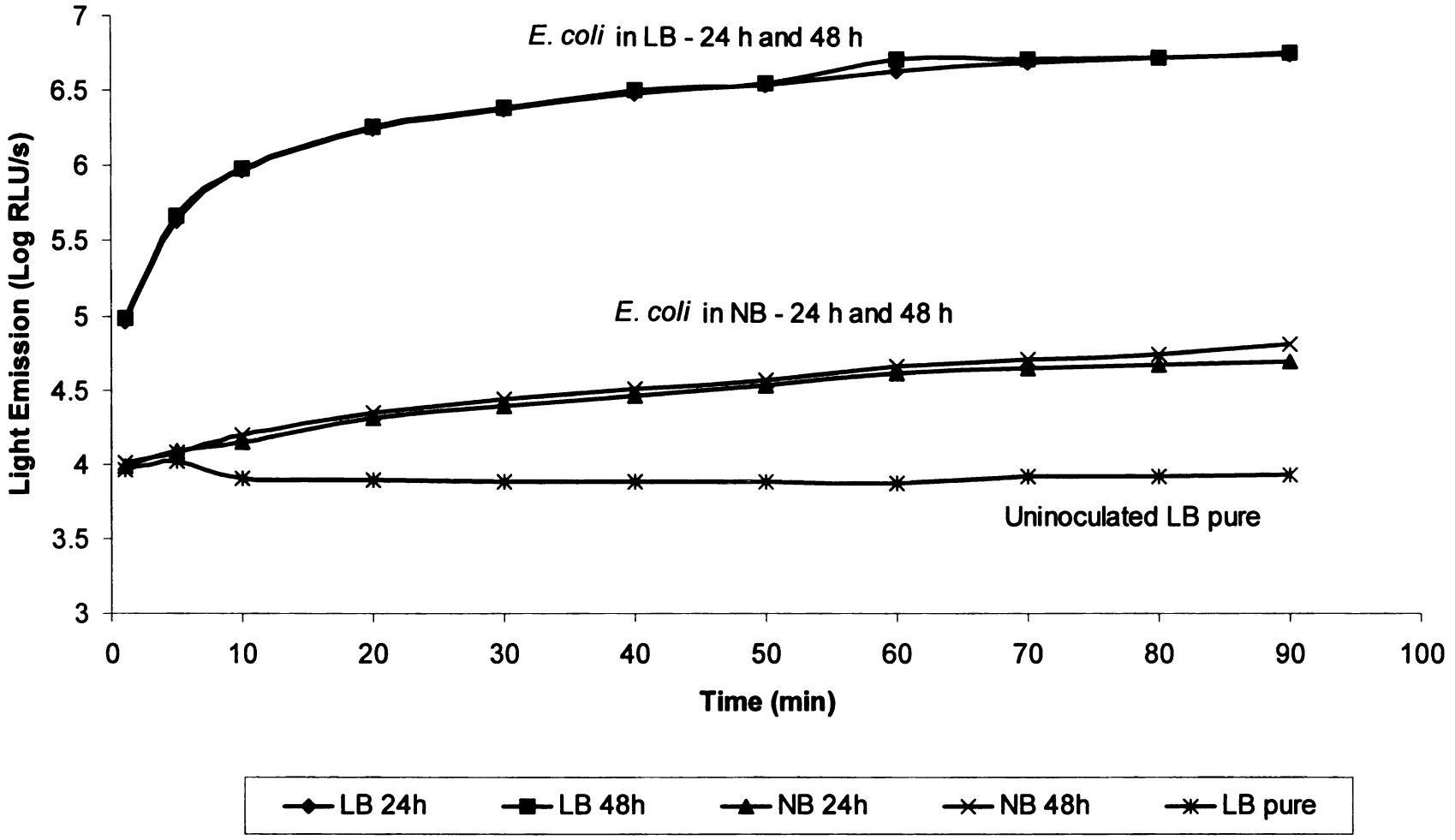

Figure 5-2. Comparative kinetics of light emitted by 24 h and 48 h old *E. coli* cultures in NB and LB.

LB were significantly different ($p<0.05$). Immediately after mixing the sample with the Lumi-Gal® 530 dioxetane substrate, the light emitted by the 24h and 48 h cultures of *E. coli* grown in LB was one log higher than that of the uninoculated LB as well as the 24 h and 48 h cultures of *E. coli* grown in NB. Light intensity for *E. coli* cultures grown in LB increased with time to 6.3 Log RLU/s with no significant increase or decrease observed after 60 min ($p>0.05$). As a result, for all later experiments, the light intensity measurement period was limited to 60 min. At the end of the light measurement period (90 min), the light emitted by the LB culture was two orders of magnitude higher than that of the NB culture ($p<0.01$) (Figure 5-2).

Tryland and Fiksdal (1998a) observed that various strains of *E. coli* required 1-4 days to reach stationary phase, thus, influencing the chemiluminescence. However, in the current study, increasing incubation time from 1 to 2 days had no significant effect on chemiluminescence. *Escherichia coli* cultures grew rapidly in both NB and LB to reach the stationary phase of growth within 24 h (as evidenced by no significant differences in *E. coli* numbers between 24 h and 48 h cultures), thus explaining the results obtained in the current study.

A 100-1000 fold increase in the light emitted by the LB *E. coli* culture in comparison to the NB culture supported the hypothesis that lactose broth would be a better medium for the assay. Availability of lactose as a substrate for *E. coli* increases β-galactosidase production. The *lac* operon in *E. coli* consists of one regulatory gene (the *I* gene) and three structural genes (*Z*, *Y*, and *A*). The *lac I* gene codes for the repressor of the *lac* operon. The *lac Z* gene codes for β-galactosidase, which is primarily responsible for the hydrolysis of lactose into its monomeric units, galactose and glucose. The *lac Y* gene

codes for permease, which increases permeability of the cell to β-galactosides. The *lac A* gene encodes a transacetylase that has a known, but not essential, enzymatic activity. During normal growth on a glucose-based medium, the *lac* repressor is bound to the operator region (*lac O*) of the *lac* operon, preventing transcription. However, in the presence of an inducer (lactose) of the *lac* operon, the repressor protein binds to the inducer and is rendered incapable of interacting with the operator region of the operon. RNA polymerase is thus able to bind at the promoter region (*lac P*), and transcription of the operon ensues, resulting in increased production of the β-galactosidase enzyme (King, 2002).

5.1.3 Determining Assay Applications

When the two *E. coli* cultures were tested with the chemiluminescence assay, no significant difference in light emission was observed between the light emissions for *E. coli* K-12 and *E. coli* O157:H7 throughout the entire 60-min period of light measurement (Figure 5-3). β-galactosidase enzyme activity for the pathogenic strain of *E. coli* was higher in LB versus that in NB, as observed with the generic *E. coli* strain grown in LB. However, with the heat-killed *E. coli* O157:H7 culture, the light emission was significantly lower than that of the untreated *E. coli* and *E. coli* O157:H7 cultures ($p<0.01$). The light intensity for the heat-killed *E. coli* O157:H7 culture was also significantly lower than that of the uninoculated broth (used as the control) ($p<0.05$) (Figure 5-3).

Lack of significant differences in light emitted for an overnight *E. coli* O157:H7 compared to *E. coli* K-12 indicates that the assay cannot be used as a discriminatory test for non-pathogenic versus pathogenic strains of *E. coli*. The use of other enzymes, such

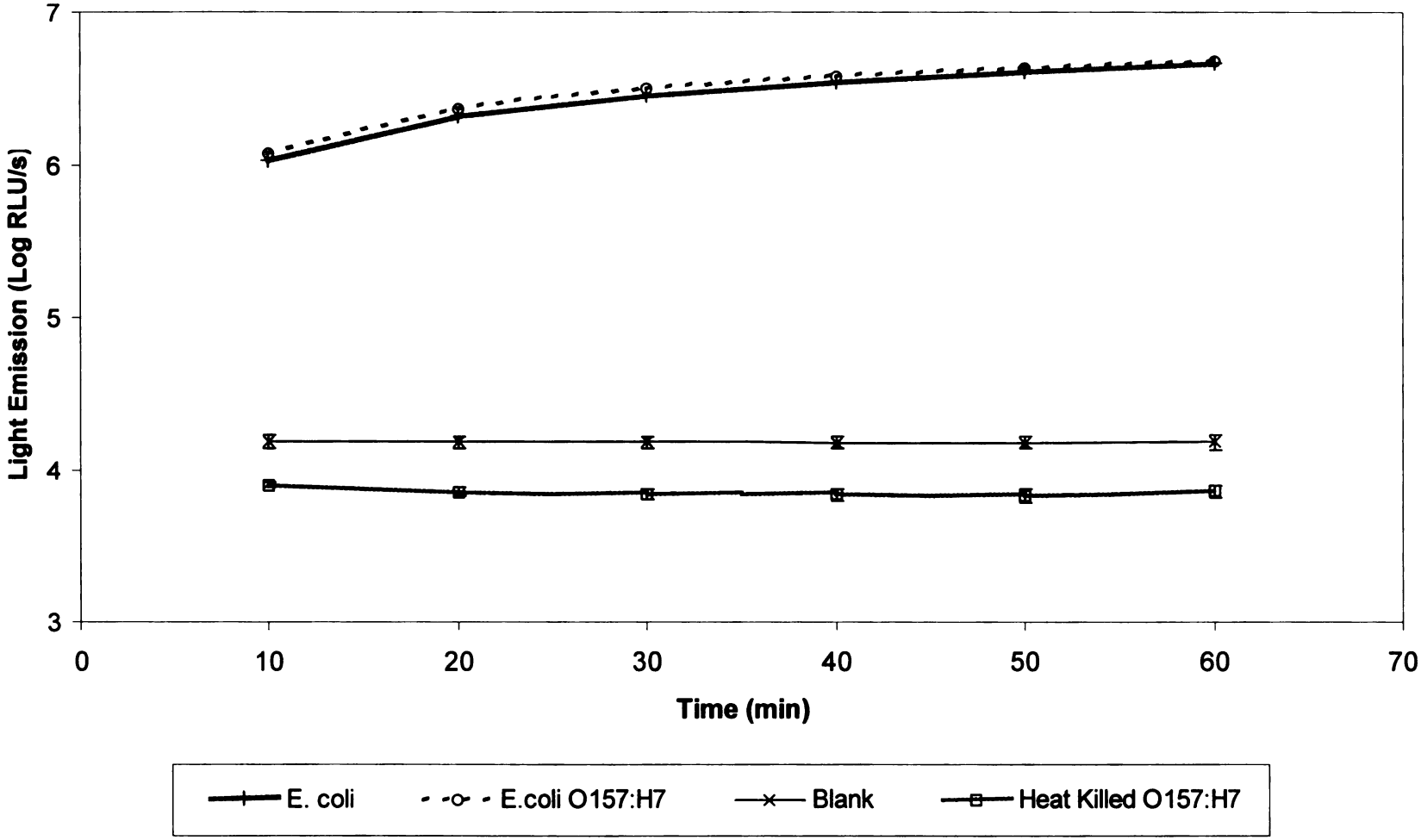

Figure 5-3. Kinetics of light emitted by an overnight generic *E. coli* and *E. coli* O157:H7 (untreated and heat-killed) culture.

as β-glucuronidase, to discriminate between non-pathogenic strains of *E. coli* and *E. coli* O157:H7 needs to be investigated. Significant differences in light emission for heat-killed *E. coli* O157:H7 cultures as compared to untreated *E. coli* O157:H7 and the LB control indicate that the assay can be used for quality control of heat-processed foods. The assay might be used to assess possible cross-contamination with raw product or ineffective heat treatment of foods such as pasteurized milk or apple cider. Significantly lower light intensity of the heat-treated sample compared to the uninoculated control could be explained by presence of organic matter (from the dead cells) in the sample. Lumi-Gal® 530 (the dioxetane substrate) has been carefully formulated to optimize enzyme activity, chemiluminescence intensity and lifetime for high sensitivity, which also makes it sensitive to the sample matrix. The organic matter present in the sample quenches the light emitted, lowering the light emission output for the heat-treated sample compared to the background signal emitted for the uninoculated control devoid of any organic matter (Beale *et al.*, 1992). This could potentially be exploited to determine presence of unusually high levels of organic matter in a food sample that also contains *E. coli* or other β-galactosidase producing organisms as a part of its normal microflora. For example, a mastitic milk sample from an infected cow normally has a high somatic cell count in addition to bacteria (Tsenkova *et al.*, 2001). Testing pasteurized mastitic milk (pooled with normal milk) could yield a chemiluminescence signal significantly lower than that of normal pasteurized milk (an untested hypothesis), which in turn can be used as an indicator of potential problems with animals in a dairy farm.

5.1.4 Determining Amount of Sample Used in the Assay

Preliminary trials conducted using 25 µl of *E. coli* culture for the assay showed a very low sensitivity of 10^5 CFU ($4x10^6$ CFU/ml for a liquid sample) after 60 min. Randomized block design (RBD) analysis comprising three levels of culture was used to optimize the assay performance in terms of sensitivity. The interactive effect of the amount of culture and the light emission was analyzed using an Autoregressive mixed model in SAS, the results of which are summarized in Table 5-1.

The intensity of chemiluminescence was directly proportional to the *E. coli* cell concentration (β-galactosidase) in the sample. With decreasing cell numbers, the light intensity decreased exponentially. For example, with 50 µl of culture, light intensity for 10^8 CFU/ml, 10^7 CFU/ml and 10^6 CFU/ml was 5.99, 5.07, and 4.38 log RLU/s, respectively, after 10 min (Table 5-1). The intensity of light emitted increased with the amount of *E. coli* culture used in the assay; for example, light emission of the 10^6 CFU/ml *E. coli* culture using 25, 50 and 55 µl was 4.3, 4.38, and 4.41 log RLU/s, respectively, after 10 min (Table 5-1).

The mean coliform count of the pure culture for the sensitivity trials was 8.19 ± 0.09 Log CFU/ml. The intensity of light emission for all samples at all serial dilution levels increased with time (10-60 min). Readings of light emission were stable after 30 min, thus 30-60 min readings were used for data analysis. The lower limit of detection based on significantly different light emission values [highlighted in Table 5-1] for 25 µl sample was 10^4 CFU at 60 min; for 50 µl culture level was 10^3-10^4 CFU between 30 to 60 min; and for 55 µl was 10^2-10^3 CFU (30-60 min). This was determined using the

Table 5-1. Light intensity for different amounts of overnight LB *E. coli* culture at various cell concentrations.

Time (min)	Culture Volume (µl)	Control (Log RLU/s)	10^3 CFU/ml (Log RLU/s)	10^4 CFU/ml (Log RLU/s)	10^5 CFU/ml (Log RLU/s)	10^6 CFU/ml (Log RLU/s)	10^7 CFU/ml (Log RLU/s)	10^8 CFU/ml (Log RLU/s)
10	25	4.18 ± 0.03^a	4.21 ± 0.02^a	4.19 ± 0.02^a	4.21 ± 0.04^a	4.30 ± 0.05^b	4.80 ± 0.05^c	5.78 ± 0.18^d
	50	4.19 ± 0.02^a	4.19 ± 0.04^a	4.22 ± 0.03^a	4.21 ± 0.03^a	4.38 ± 0.06^b	5.07 ± 0.05^c	5.99 ± 0.07^d
	55	4.20 ± 0.02^a	4.21 ± 0.03^a	4.20 ± 0.02^a	4.22 ± 0.03^b	4.41 ± 0.06^c	5.10 ± 0.05^d	6.05 ± 0.06^e
20	25	4.18 ± 0.03^a	4.20 ± 0.03^a	4.19 ± 0.03^a	4.21 ± 0.04^a	**4.37 ± 0.05^b**	5.04 ± 0.05^c	6.10 ± 0.19^d
	50	4.18 ± 0.02^a	4.20 ± 0.04^a	4.20 ± 0.02^a	4.23 ± 0.03^a	**4.52 ± 0.06^b**	5.32 ± 0.05^c	6.27 ± 0.07^d
	55	4.19 ± 0.03^a	4.20 ± 0.02^a	4.22 ± 0.02^a	**4.24 ± 0.03^b**	4.56 ± 0.07^c	5.37 ± 0.05^d	6.33 ± 0.06^e
30	25	4.18 ± 0.03^a	4.19 ± 0.04^a	4.19 ± 0.04^a	4.22 ± 0.03^a	**4.43 ± 0.05^b**	5.17 ± 0.06^c	6.19 ± 0.18^d
	50	4.18 ± 0.03^a	4.20 ± 0.03^a	4.19 ± 0.04^a	**4.25 ± 0.04^b**	4.60 ± 0.07^c	5.46 ± 0.05^d	6.41 ± 0.07^e
	55	4.19 ± 0.02^a	4.19 ± 0.02^a	**4.23 ± 0.03^b**	4.26 ± 0.02^b	4.64 ± 0.06^c	5.51 ± 0.05^d	6.46 ± 0.05^e

Table 5-1 (cont'd).

Time (min)	Culture Volume (µl)	Control (Log RLU/s)	10^3 CFU/ml (Log RLU/s)	10^4 CFU/ml (Log RLU/s)	10^5 CFU/ml (Log RLU/s)	10^6 CFU/ml (Log RLU/s)	10^7 CFU/ml (Log RLU/s)	10^8 CFU/ml (Log RLU/s)
40	25	4.17±0.03^a	4.19±0.04^a	4.19±0.05^a	4.22±0.04^a	**4.47±0.05^b**	5.26±0.06^c	6.29±0.18^d
	50	4.18±0.02^a	4.19±0.03^a	4.20±0.04^a	**4.26±0.03^b**	4.66±0.07^c	5.55±0.06^d	6.50±0.07^e
	55	4.19±0.03^a	4.20±0.02^a	4.24±0.03^{ab}	**4.28±0.02^{bc}**	4.71±0.07^d	5.60±0.06^e	6.56±0.06^f
50	25	4.17±0.03^a	4.19±0.04^a	4.19±0.05^a	4.22±0.04^a	**4.50±0.06^b**	5.32±0.06^c	6.35±0.18^d
	50	4.18±0.03^a	4.19±0.04^a	4.21±0.03^a	**4.27±0.03^b**	4.71±0.07^c	5.62±0.06^d	6.56±0.07^e
	55	4.19±0.02^a	4.19±0.02^a	**4.25±0.02^b**	4.29±0.03^c	4.77±0.08^d	5.66±0.06^e	6.62±0.05^f
60	25	4.18±0.03^a	4.20±0.02^a	4.20±0.04^a	4.24±0.04^a	**4.55±0.05^b**	5.38±0.05^c	6.41±0.17^d
	50	4.19±0.02^a	4.20±0.03^a	4.22±0.04^a	**4.30±0.03^b**	4.76±0.06^c	5.68±0.05^d	6.62±0.07^e
	55	4.19±0.03^a	4.20±0.02^a	**4.26±0.02^b**	4.32±0.02^c	4.80±0.08^d	5.72±0.05^e	6.67±0.05^f

Means in the same row with different superscript are significantly different ($p<0.05$). Values in bold indicate the point at which the significant differences begin.

equation: Sensitivity (CFU) = [Average coliform count (in CFU/ml corresponding to bold light emission value) x volume of sample (µl)]/1000 µl.

The sensitivity of the assay was observed to be low (10^5 CFU) using 25 µl of *E. coli* culture, resulting in the investigation of different levels of the culture to maximize the sensitivity. As recommended by Lumigen (the supplier), Lumi-Gal® 530 was not to be diluted by more than 10% of the total volume with the culture or enzyme. The three *E. coli* culture amounts – 25, 50 and 55 µl - were chosen accordingly. Maximum sensitivity of the assay required β-galactosidase and stabilized dioxetane to be mixed and incubated for sufficient time to allow the product (detected by luminometer) to approach steady state. About 30 min after mixing Lumi-Gal® 530 and the permeabilized culture mix, the light emissions were stable; therefore, the readings from 30-60 min were considered in the interpretation of results. Beale *et al.* (1992) followed a similar procedure while testing Lumi-Gal® 530 with purified β-galactosidase. The enzyme was mixed with Lumi-Gal® 530, followed by sample incubation at 37°C for 30 min and then the chemiluminescence response was measured (Beale *et al.*, 1992).

Sensitivity of the assay improved with the increasing amount of *E. coli* culture used for the assay. Higher cell numbers, and thus higher amount of β-galactosidase enzyme, in 55 µl of *E. coli* culture may explain maximum sensitivity of 10^3 CFU. This means that any sample analyzed (using 55 µl) had to contain a minimum of 10^3 CFU in the reaction mixture for successful detection. Thus, the assay would require filtration or similar processes to concentrate the cells for samples with low numbers (10^0-10^2 CFU). An enrichment process to elevate the *E. coli* or coliform numbers to detectable levels could also be used. Tryland and Fiksdal (1998b) used isopropyl-β-D-thiogalactopyranoside

(IPTG), a β-galactosidase inducer, and observed that the β-D-galactosidases of all non-target environmental isolates were less inducible than the enzymes of coliforms and *E. coli* when grown in tryptic soy broth. So, the addition of β-galactosidase inducers, such as IPTG, to LB during an enrichment step could increase β-galactosidase activity of *E. coli* in samples contaminated at low levels, potentially reducing enrichment time required and improving the sensitivity of the assay. This is yet to be investigated.

5.1.5 Assay Validation in Food Analysis

Based on the results of the sensitivity trials, a sample of sprouts (SS), three samples of milk (M1, M2, M3), and three samples of water (W1, W2, W3) inoculated with *E. coli* were tested using 55 µl sample and polymixin B sulfate mixture with 500 µl Lumi-Gal® 530. *Escherichia coli* levels below 10^3 CFU/ml could not be detected in the samples tested (Figure 5-4) as the difference in intensity of light emitted between the samples and the blank was not significantly different ($p>0.05$). At *E. coli* levels above 10^3 CFU/ml, the intensity of light emitted increased exponentially with the bacterial numbers. Moreover, the sample matrix did not have any significant effect on the assay.

Figure 5-5 shows the light output measured 60 min after addition of Lumi-Gal® to the Red Cedar River water samples collected from seven sites. Based on analysis of the sensitivity study, a guideline was used to assess the presence or absence of coliforms in a sample with unknown number of coliforms. If the light emission from the sample was consistently greater than that of the blank/control by at least 1000 RLU/s (between 30-60 minutes), the sample was considered positive. Based on this guideline, when the water samples were analyzed directly (without pre-enrichment), a conclusive

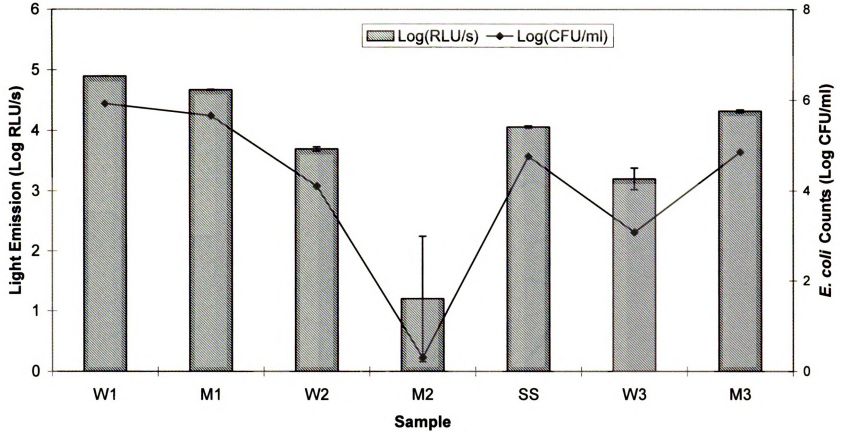

Figure 5-4. Normalized light emission and *E. coli* counts of food and water samples. Key: W - water, M - milk, SS - alfalfa sprout seeds. *Normalized = (Sample - Blank) in RLU/s.*

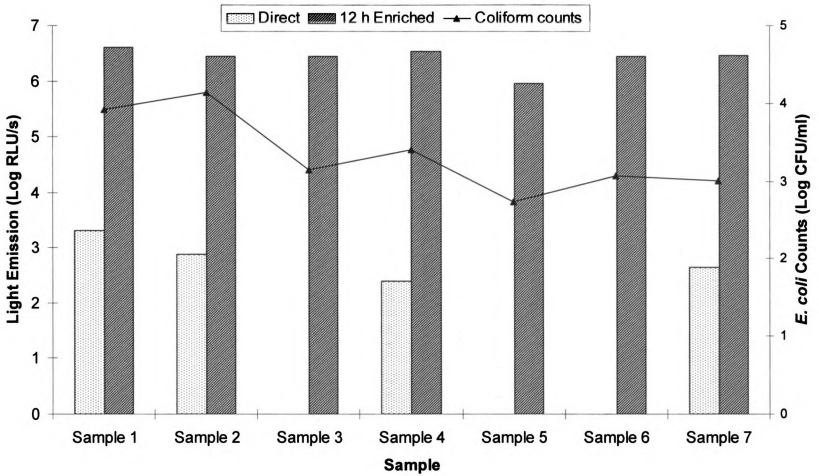

Figure 5-5. Normalized light emission of surface water samples direct assay and 12 h enrichment in LB prior to assay. Sample coliform counts are prior to enrichment. *Normalized = (Sample – Blank) in RLU/s.*

interpretation of the data (presence/absence and/or quantification) could not be made for five of the seven samples. Only sample 1 and sample 2 could be interpreted as positive. Subsequently, the coliform counts for samples 1 and 2 (from MacConkey agar plates) were found to be ~1 log higher than the rest of the samples; counts ranged from 550 CFU/ml (for sample 5) to 13,500 CFU/ml (for sample 2). Light emission from samples 3, 5, and 6 was very close to that of the blank (Figure 5-5). When the assay was repeated following enrichment in LB for 1-6 h, no significant difference in light emission was observed between enriched samples and those without enrichment (data not shown). However, after a 12-h enrichment of all samples in LB, the light emission increased by 2.5 orders of magnitude for all samples compared to the direct assay. Interpretation of results from the 12-h enrichment assay was conclusive in detecting presence/absence of coliforms in all the samples (compared with plate counts).

When surface water samples, contaminated at low levels (10^1-10^3 CFU/ml), were analyzed using the assay without any pre-enrichment in LB, the assay indicated a presumptive positive/negative result. This was due to the stationary (or lag) phase condition of the *E. coli* cells in the water samples. Production of β-galactosidase by *E. coli* is affected by several factors, such as cell growth phase, availability of nutrients like glucose versus lactose in the growth medium, ATP levels in the *E. coli* cells, strain of *E. coli*, all of which influence the amount of enzyme that can be detected definitively by the assay procedure.

When enriched in LB, at first the *E. coli* cells are quiescent; they do not metabolize the lactose, their other metabolic activities decline, and cell division ceases. During the quiescent interval, the cells begin to produce three enzymes (with transcription of the *lac*

operon gene as explained above) in large amounts that have not been previously produced. The culture then begins growing rapidly again with the lactose being rapidly consumed (Adhya, 1996; Kimball, 2003). This growth behavior during enrichment explains the inability to obtain significantly higher light emission with samples enriched for 1-6 h.

In conclusion, the development of a chemiluminescence-based assay and its application in food samples for detection of coliforms (*E. coli*), outside of the biosensor system has been demonstrated. The detection limit of the assay was 10^3 CFU. The assay was completed within 60 min. This assay will be adapted to the biosensor system in the third research objective.

5.2 OBJECTIVE 2

Design, fabrication and characterization of a nano-tubular silicon platform.

5.2.1 Nano-Tubular Si Characterization

NTS was fabricated from a p^{++} type wafer using different anodization conditions. These conditions were chosen by modifying those used for porous silicon fabrication (Shimasaki *et al.*, 1996; Thust *et al.*, 1996; Halimaoui, 1997) reported previously. Scanning electron microscope (SEM) images (Figures 5-6 to 5-13) of the chips were used to determine the best NTS biosensor fabrication conditions based on nano-tube size, shape and uniformity, nano-tubular surface texture and inter-nano-tube space. No features were observed on the bare (non-nano-tubular) Si (Figure 5-6). NTS fabricated using anodization conditions of 2 mA/cm^2 for 1 h (Figure 5-7) showed a uniform structure, slightly uneven surface texture with noticeably less inter-nano-tube spaces (5-20 nm), and nano-tube size ranging from 5–30 nm. Anodization conditions of 5 mA/cm^2 for 20 min

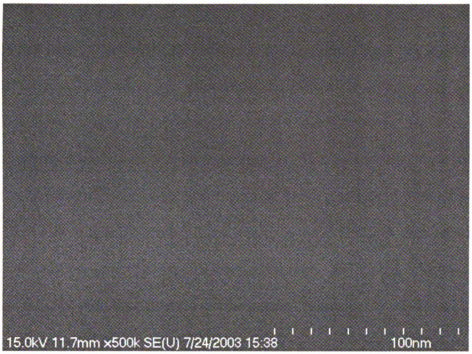

Figure 5-6. SEM image of bare p^{++} Si surface.

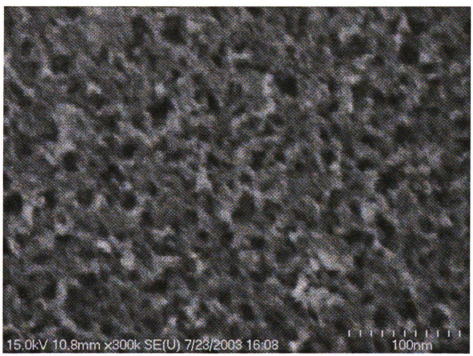

Figure 5-7. SEM image of nano-tubular Si produced from p++ type Si. Anodizing conditions used were 15% HF, 2 mA/cm2 for 1 h.

(Figure 5-8) and 5 mA/cm^2 for 1 h (Figure 5-9) provided the best NTS chip characteristics with uniform nano-tube structure and nano-tube sizes ranging from 10-30 nm, a smooth (even) surface, and an inter-nano-tube space of 10-20 nm.

NTS fabricated using 15 mA/cm^2 (Figure 5-10) and 30 mA/cm^2 for 1 min (Figure 5-11) showed an uneven surface texture and non-uniform structures. Nano-tubes were elliptical or rod-shaped with large inter-nano-tube spaces and nano-tube sizes ranging from 5–20 nm in length. NTS fabricated using 50 mA/cm^2 for 1 min (Figure 5-12) showed non-uniform structures, highly uneven texture, and round, elliptical or rod-shaped nano-tubes with less inter-nano-tube spaces compared to 15 mA/cm^2 and 30 mA/cm^2. Nano-tube sizes ranged from 5–30 nm in length.

All the properties of a NTS layer are strongly dependent on anodizing conditions such as HF concentration, pH of the solution and its chemical composition, current density, wafer type and resistivity, temperature, anodization duration, and illumination (Halimaoui, 1997). NTS fabricated using 100 mA/cm^2 for 30 s (Figure 5-13) had a poor and uneven surface structure. Uneven shaped nano-tubes with very large inter-nano-tube size spaces were observed. Visual inspection of the NTS chip showed discoloration as well as partial etching of the top NTS surface. This result contradicted previously reported literature where porous silicon was successfully fabricated using 100 mA/cm^2 for 30 s to 1 min (Halimaoui, 1997; Janshoff *et al.*, 1998). This discrepancy could be due to variations in the resistivity of the wafers used and the concentration of the HF solution.

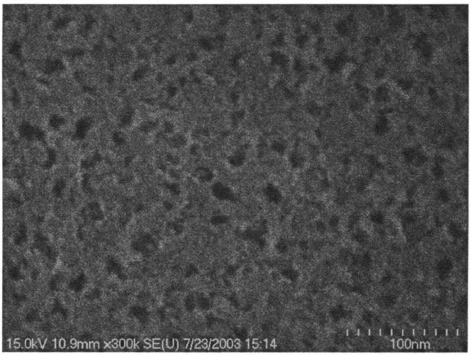

Figure 5-8. SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 5 mA/cm^2 for 20 min.

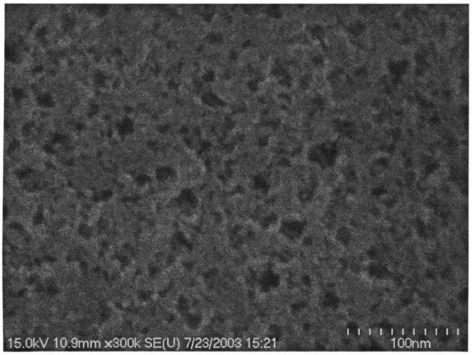

Figure 5-9. SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 5 mA/cm^2 for 1 h.

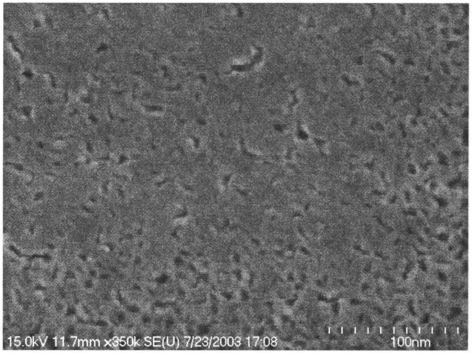

Figure 5-10. SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 15 mA/cm^2 for 1 min.

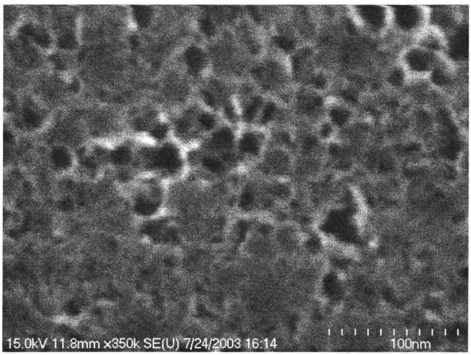

Figure 5-11. SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 30 mA/cm^2 for 1 min.

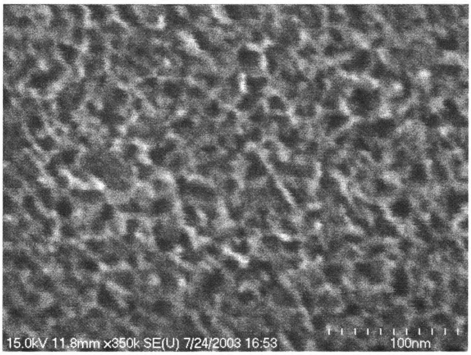

Figure 5-12. SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 50 mA/cm^2 for 1 min.

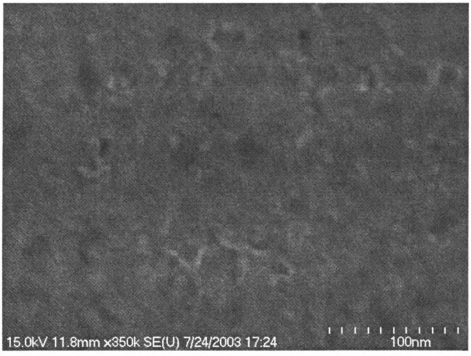

Figure 5-13. SEM image of nano-tubular Si produced from p^{++} type Si . Anodizing conditions used were 15% HF, 100 mA/cm^2 for 30 s.

Based on the characterization of the NTS using SEM images, 5 mA/cm^2 for 1 h was chosen for the fabrication of the NTS biosensor chip in further testing. Anodizing conditions of 5 mA/cm^2 for 1 h was chosen over 5 mA/cm^2 for 20 min because longer anodization with the same current density yields a thicker NTS layer (longer nano-tubes) (Halimaoui, 1997) and thus more surface area for the biological sensing element to bind and for the biochemical reaction during the detection process.

The porosity and thickness of the NTS obtained using anodization conditions of 5 mA/cm^2 for 1 h with 15% ethanoic solution of HF was determined gravimetrically (Table 5-2). The percent porosity of the NTS ranged from 70.00 % (Chip 10) to 87.23 % (Chip 4), and the thickness of the NTS layer varied from 13.09 μm (Chip 3) to 21.67 μm (Chip 1). The mean percent porosity of the NTS layer for the 14 chips was 80.21 % ± 4.29 % with a corresponding mean thickness of 17.54 μm ± 2.82 μm. The percent porosity of the NTS was similar to that reported previously under similar anodizing conditions (Halimaoui, 1997; Janshoff *et al.*, 1998; Amato *et al.*, 2000). The variation in porosity and thickness observed was possibly due to batch-to-batch variations in composition of the ethanoic HF used or slight fluctuations in the current density applied to the silicon chip.

A cross-sectional SEM image was taken by carefully dicing the NTS chip with a diamond saw and mounting the cross-section of the chip at a 45° angle. The scanning electron micrograph provided additional confirmation of the thickness obtained by gravimetric analysis (Figure 5-14). The micrograph shows the NTS structures to be about 15-20 μm thick/deep and interconnected to some degree.

Table 5-2. Thickness and porosity of nano-tubular Si chips fabricated from p^{++} Si.

Chip	m_1 (g)	m_2 (g)	M_3 (g)	Area (cm^2)	Thickness (μm)	Porosity (%)
1	0.2747	0.2708	0.2699	0.950714	21.67	81.25
2	0.3365	0.3329	0.3322	0.950714	19.41	83.72
3	0.3697	0.3674	0.3668	0.950714	13.09	79.31
4	0.32	0.3159	0.3153	0.950714	21.22	87.23
5	0.345	0.3413	0.3406	0.950714	19.86	84.09
6	0.2981	0.2946	0.2938	0.950714	19.41	81.40
7	0.3369	0.3336	0.3327	0.950714	18.96	78.57
8	0.3306	0.328	0.3272	0.950714	15.35	76.47
9	0.3087	0.3062	0.3055	0.950714	14.45	78.12
10	0.3171	0.315	0.3141	0.950714	13.54	70.00
11	0.3281	0.3249	0.3242	0.950714	17.61	82.05
12	0.3523	0.3491	0.3484	0.950714	17.61	82.05
13	0.3084	0.3059	0.3051	0.950714	14.90	75.76
14	0.2673	0.2639	0.2632	0.950714	18.51	82.93

Anodizing conditions used were 15% HF, 5 mA/cm^2 for 1 h.

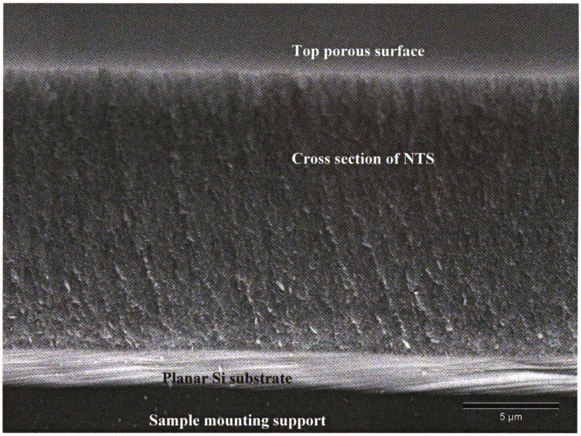

Figure 5-14. Cross-sectional SEM image of nano-tubular Si produced from p^{++} type Si. Anodizing conditions used were 15% HF, 5 mA/cm^2 for 1 h. NTS chip cross-section was placed at 45° to capture the image.

When using doped p-type silicon to produce a porous silicon-based bioreactor for glucose detection, Bengtsson *et al.* (2000a) produced a porous surface with a maximum thickness of 10 µm, while DeLouise and Miller (2005) could only obtain a 6.5 µm thick porous layer for enzyme immobilization. Other investigators have also reported similar thickness when fabricating porous silicon layers from highly doped p-type wafers (Splinter *et al.*, 2001; Martin-Palma *et al.*, 2004). Halimaoui (1997) predicted the possibility of producing a porous silicon layer 13 µm thick using 25% HF and a fixed current density of 50 mA/cm^2 for 4 min. However, a porous silicon layer with more than 10 µm thickness using highly doped p-type silicon has not been reported, especially with

lower current densities. The current research has shown a significant improvement (50-100 %) in the thickness of the porous layer over currently reported values, thus justifying the name NTS. This improvement was made possible by maintaining the potential, V, less than V_{ep}, the electropolishing peak potential. As long as the current density, $J < J_{ep}$, the supply of holes was restricted to maintain the H-terminated surface throughout etching (Chazalviel *et al.*, 2000; Safi *et al.*, 2002).

Ethanoic HF solution was used as the electrochemical etching agent, as opposed to purely aqueous HF solution, because ethanol had been shown to act as a surfactant, improving the porous silicon layer uniformity by eliminating hydrogen bubbles (formed during the process of porous silicon layer formation), improving the penetrability of the electrolyte (etching) solution in the pores (Halimaoui, 1997). Also, the use of the ethanoic HF enabled fabrication of porous silicon at higher currents for longer times than that used typically with diluted aqueous HF solutions. Longer anodization time with the same current density had been shown to provide a thicker (deeper) porous silicon layer (Halimaoui, 1997) and thus more surface area for the biochemical reactions during the detection process. When anodization times greater than 60 min were employed to fabricate NTS, the process was not repeatable; a porous layer of silicon was obtained in some trials while electropolishing occurred in others (data not shown). As the anodization time was increased, current density, J, eventually peaked beyond J_{ep}, leading to electropolishing (complete etching of the silicon surface layers) instead of pore formation (Allongue, 1997). Hence, an anodization condition of 5 mA/cm^2 for 60 min was used to fabricate the NTS platform for the biosensors, yielding porous silicon layers consistently with the maximum depth of the pores possible (before electropolishing occurred).

5.3 OBJECTIVE 3

Functionalization of the nano-tubular silicon platform into a biosensor chip.

5.3.1 Adaptation of CL Assay to the Biosensor Detection System - Procedure A

Figure 5-15 shows the light emission of the control NTS biosensor chip (blank with no *E. coli* culture) as well as that of NTS- and planar Si biosensor chips tested with an overnight *E. coli* pure culture. Light emission for the control functionalized NTS biosensor chip (with no *E. coli*) was significantly lower ($p<0.01$) than that for both the planar Si and the NTS biosensor chips, thus indicating that both chips were able to detect *E. coli* present in the sample using β-galactosidase produced as a marker (biological sensing element). However, light emission for the nano-tubular biosensor chip was significantly (about three times) higher than that of the planar biosensor chip. For example, light emission of the control biosensor chip, the planar Si chip and the NTS biosensor chip after 30 min were 5480 RLU/s, 173,666 RLU/s and 561,868 RLU/s, respectively. The higher light emission of the NTS biosensor chip as compared to the planar Si biosensor chip was due to the higher surface area available for the Lumi-Gal® 530 to diffuse and bind to during the functionalization step. With greater amounts of Lumi-Gal® 530 available to react with β-galactosidase in the sample, the amount of light emitted from the NTS biosensor chip, when compared with that of the planar Si chip, was higher.

The external CL assay (Objective 1, Table 5-1 on page 127) had higher light emission than that of the NTS biosensor chip for an *E. coli* pure culture by about one log RLU/s. This can be explained by the lower amounts ($1/10^{th}$) of sample and Lumi-Gal® 530 used in the biosensor system as compared to that of the single-tube external CL of assay. However, this also reduces the cost of the assay by 90%. Also, per unit volume

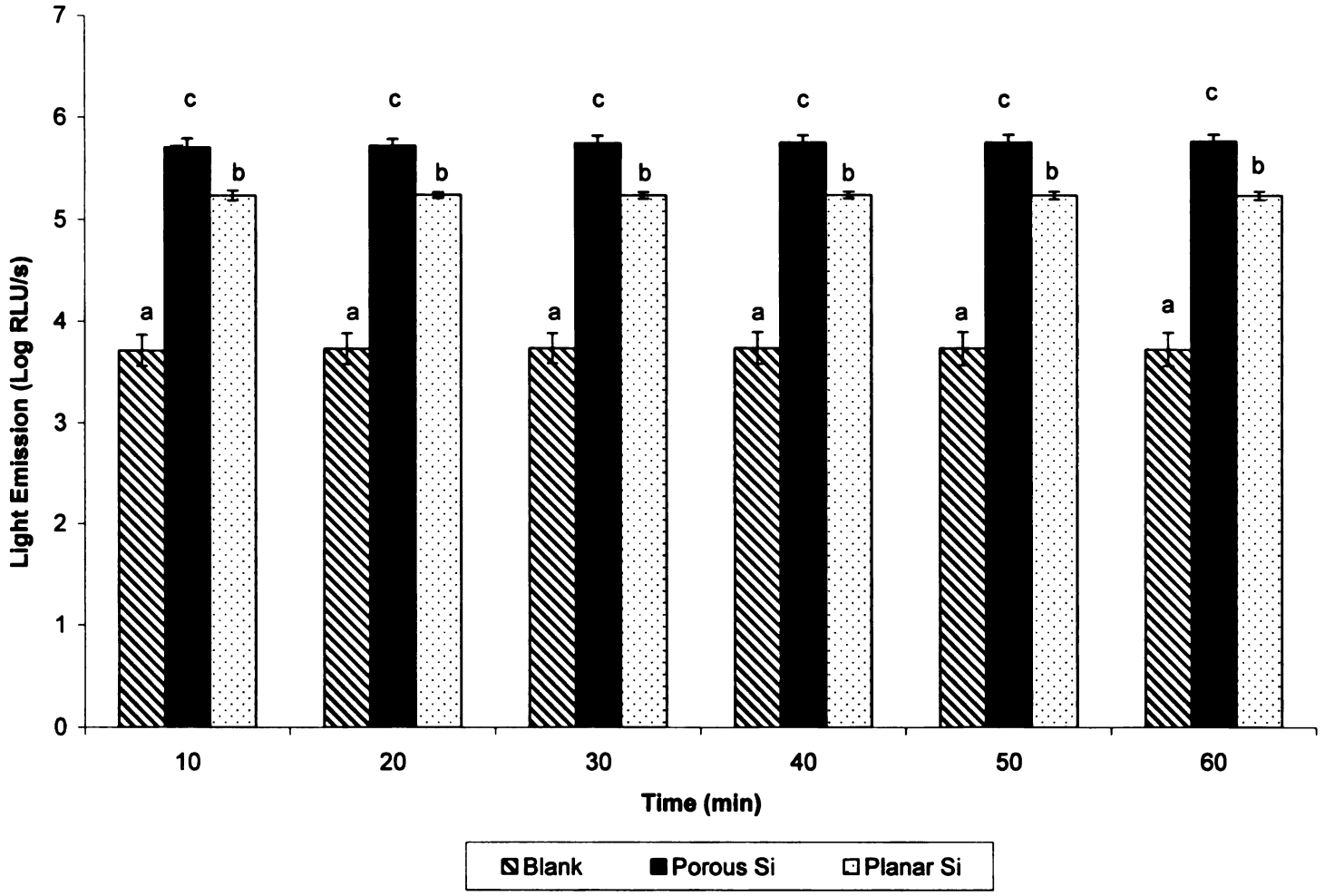

Figure 5-15. Comparison of light emission of *E. coli* pure culture in nano-tubular Si and planar silicon biosensors against nano-tubular Si control. Different superscript over the bars indicates significantly different light emission ($p<0.01$).

dioxetane substrate, light emitted from the NTS biosensor was slightly higher (4.05 Log RLU) than that from the single-tube assay (3.97 Log RLU).

The single-tube external CL-based assay developed in Objective 1 (sections 5.1.1 to 5.1.5) was successfully adapted to the NTS biosensor chip system to detect *E. coli* using β-galactosidase as the biological sensing element. The NTS biosensor platform is practically an array of single-tube external assays in the nano scale.

5.3.2 Adaptation of CL Assay to the Biosensor Detection System - Procedure B

Figure 5-16 shows the comparative kinetics of light emission for an overnight *E. coli* culture grown in LB using the standard chemiluminescence assay, developed previously, and the modified chemiluminescence assay where the Polymyxin B sulfate and dioxetane substrate were mixed prior to addition of the *E. coli* culture. Mean light emission for *E. coli* with the modified chemiluminescence assay was equivalent to that obtained with the standard chemiluminescence assay immediately after addition of the *E. coli* culture to the dioxetane-Polymyxin B sulfate mixture. However, an incubation period of 15 min after addition of culture to the Polymyxin-dioxetane mixture significantly improved the light emission over the standard chemiluminescence assay procedure. Light emission for the modified chemiluminescence assay procedure (after incubation) was significantly greater than the standard assay throughout the 60 min period of measurement ($p<0.01$) (Figure 5-16).

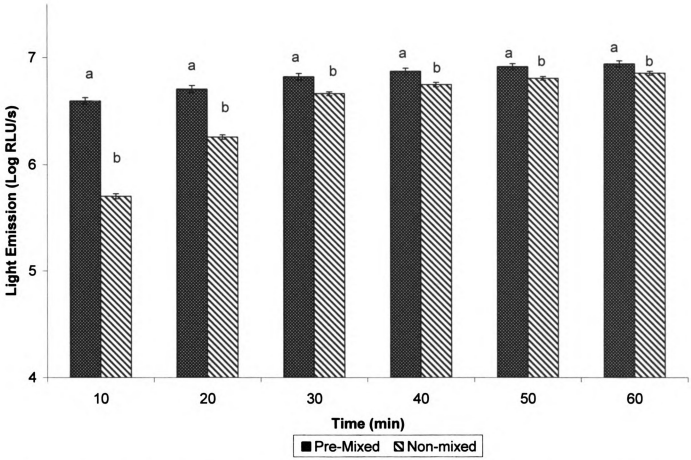

Figure 5-16. Kinetics of light emitted by an overnight LB *E. coli* culture using the standard (non-mixed) and modified (pre-mixed with 15 min incubation) single-tube chemiluminescence assays. Different superscripts over the bars indicate significant differences ($p<0.01$).

Figure 5-17 shows the light emission from serially diluted *E. coli* cultures on NTS biosensor chips. With increasing cell numbers, the light intensity also increased. For example, light intensity of samples containing 10^1 CFU, 10^2 CFU, 10^3 CFU and 10^4 CFU of *E. coli* were 3.38, 3.52, 3.85 and 4.22 log RLU/s, respectively, at the 40 min reading (Figure 5-17). The amount of β-galactosidase in the sample extracted from the cells would be higher with increasing *E. coli* cell numbers, thus leading to a greater chemiluminescence intensity. Beale *et al.* (1992) also observed a linearly increasing relationship between β-galactosidase concentration and chemiluminescence using Lumi-Gal® 530.

Following addition of the sample to the functionalized biosensor chips, light emission increased with time and had lower variability after 30 min. This trend was similar to that observed previously with the single-tube chemiluminescence assay external to the biosensor (Mathew and Alocilja, 2004) and the study by Beale *et al.* (1992). Hence, light emission values obtained after 30 min of sample addition in this study were used to determine the sensitivity of the biosensor to *E. coli*.

The average bacterial count of the *E. coli* pure culture for the sensitivity trials was 5.66×10^7 CFU/ml. The lower limit of detection based on significantly different light emission values was 10^2 CFU at 30 and 50 min and 10^1 at 40 and 60 min. This was determined using the same equation as before: Sensitivity (CFU) = [Average coliform count (in CFU/ml corresponding to bold light emission value) x volume of sample (μl)]/1000 μl. The lower detection level of 10^1-10^2 CFU was a significant improvement over that of the single-tube chemiluminescence assay determined external to the biosensor (10^3 cells, (Mathew and Alocilja, 2004)). This improvement could be explained

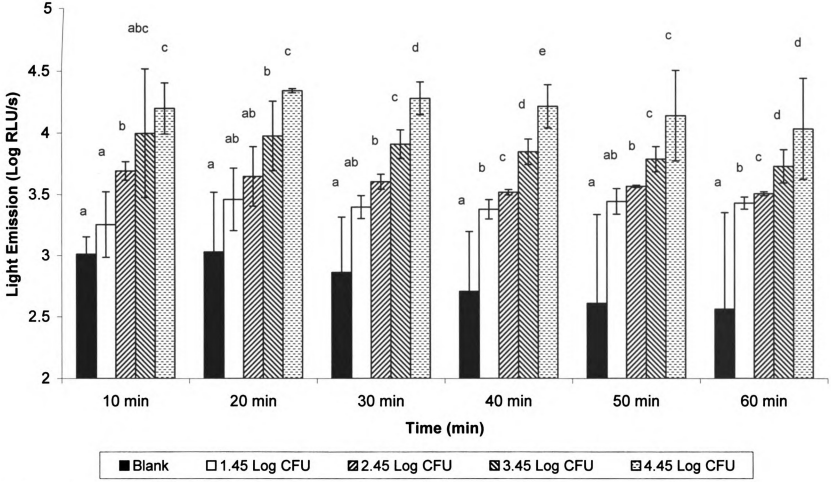

Figure 5-17. Comparison of light emission from serially diluted *E. coli* cultures on nano-tubular Si biosensor against nano-tubular Si control (blank). Different superscript over the bars indicates significantly different light emission ($p<0.05$).

by the lower background noise observed with the control sample (10^2-10^3 RLU/s, Figure 5-16) for the modified chemiluminescence assay on the biosensor chip as compared to the background light emission for the single-tube chemiluminescence assay (10^4 RLU/s). With lower background noise, the assay could detect smaller differences in light emission that resulted from samples contaminated with low numbers of *E. coli*, thus improving the sensitivity of the assay.

In conclusion, functionalization of the NTS into a biosensor was achieved using diffusion and physical adsorption of the dioxetane substrate as confirmed by a chemiluminescence-based assay with β-galactosidase produced by *E. coli* as a marker. Light emission of the NTS biosensor chip was higher than that of the planar Si biosensor chip. *Escherichia coli* could be detected using the modified assay procedure within 30 min after addition of the sample to the functionalized NTS biosensor chip. The lower detection limits (sensitivity) of the biosensor for *E. coli* were 10^1 and 10^2 cells at 40 and 30 min, respectively, which was an improvement over the sensitivity of 10^3 cells for the single-tube chemiluminescence assay developed previously. Future research will involve adapting the biosensor to detect disease-causing agents, such as *Salmonella*, *E. coli* O157:H7 and *Listeria*.

5.3.3 Functionalization of the Nano-Tubular Si Platform with *Salmonella* Antibodies into an Immuno-Biosensor

The NTS surface was functionalized into an immuno-biosensor by physical adsorption of antibodies as well as a combination of silanization and antibody immobilization processes. The biosensor chips were then used to successfully detect *Salmonella*.

Figure 5-18 shows the light emission of a control NTS biosensor chip (with no *Salmonella*) as well as that of nano-tubular- and planar silanized Si biosensor chips tested with an overnight culture of *Salmonella*. The average bacterial count determined by standard plating was 8.68 ± 0.23 Log CFU/ml of *Salmonella*. The intensity of light emitted for the NTS biosensor chip with *Salmonella* increased for the first 2 minutes and then decreased slowly while that of the control and planar Si chip decreased slowly over the entire 10 min period of light measurement. Light emission from the NTS chip was higher than that of the planar Si chip throughout the measurement period. However, the difference in light emission was not statistically significant ($p>0.05$) due to high variability in light emission for the NTS chip (coefficient of variance ranging from 2.04 to 8.92% for NTS chip as compared to 0.38 to 1.48% for planar silicon chip). Standardization of biosensor functionalization steps and improving the efficiency of blocking/washing steps during chemiluminescence assay can reduce this variability. However, there may not be a significant advantage when using the NTS biosensor chip as an immuno-biosensor because the size of the bacterial target (approximately 0.5 x 3 μm in diameter or length) exceeds the size of the nano-tubes greatly, thus reducing the chances of penetrability of the target into the nano-tubes. Overall, light emission of the nano-tubular and planar silicon chips was significantly greater than that of the control chip ($p<0.05$), indicating presence of *Salmonella* in the sample.

However, the NTS could easily be adapted to detect viruses of significance to human health. Viral particles are much smaller than the bacterial agent used in the current research, and are reported to have a particle size ranging from 20-250 nm or greater depending on the size of the single- or double-stranded RNA or DNA genome

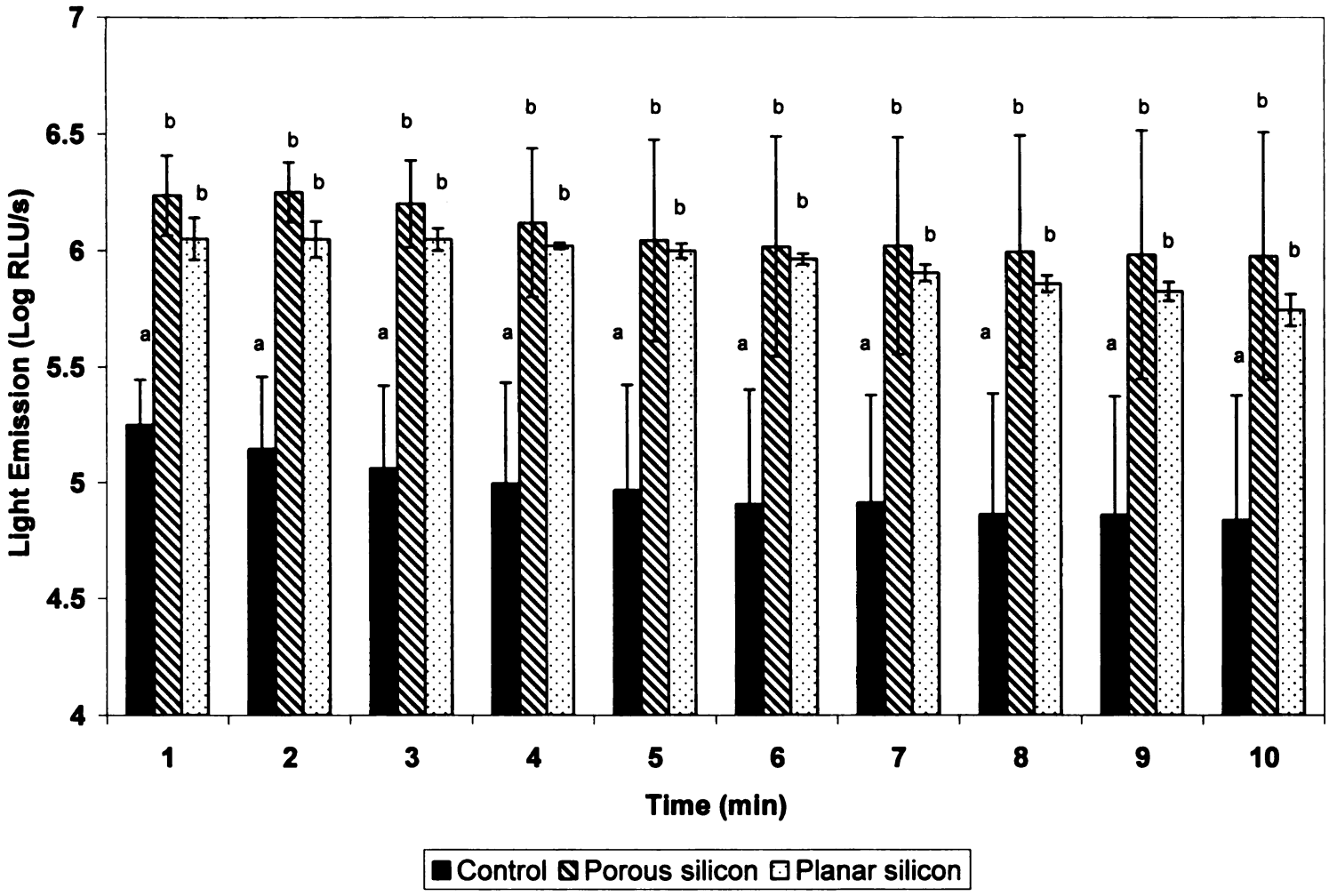

Figure 5-18. Comparison of light emission for *Salmonella* pure culture with nano-tubular and planar silanized Si biosensor against nano-tubular silanized Si control. Different superscript over bars indicates significantly different light emission ($p<0.05$).

(Balch *et al.*, 2000; Yamaguchi *et al.*, 2003; Brussaard *et al.*, 2004). Macroporous (up to 1 μm diameter pores) NTS-based immuno-biosensor can be fabricated by using appropriate anodization conditions to detect the larger sized viral particles. Another possible medical application for the NTS immuno-biosensor would be detection of antibodies produced against viral and bacterial targets in blood samples (Yamaguchi *et al.*, 2003). The NTS biosensor can then be used as a nano-biosensor device for detection of nano particles to provide increased speed and sensitivity in biological sensing applications, compared to their macro or micro counterparts (Ilic *et al.*, 2004; Lee and Chang, 2005).

Light emission of the control NTS biosensor chip (with no *Salmonella*) was also compared against that of silanized and non-silanized (physically adsorbed antibodies) NTS biosensor chips tested with an overnight *Salmonella* pure culture (Figure 5-19). Light emission of the control chip and the non-silanized chip tested with *Salmonella* was approximately one log lower than that of the silanized NTS chip with *Salmonella*. For example, light emitted from the control, non-silanized and silanized chips after 5 min were 4.97, 5.09, and 6.04 Log RLU/s, respectively. Light emission for the *Salmonella* pure culture on the silanized NTS chip was significantly greater ($p<0.05$) than the control as well as the non-silanized NTS chip with *Salmonella* for all time measurements, indicating successful silanization and detection of *Salmonella*.

HRP-Luminol® reaction kinetics showed an exponential decrease in light emission with the light remaining intense for about 20 minutes (data not shown). Based on reaction kinetics, the half-life of the HRP-Luminol® reaction was reported to be 60 min (Pierce Chemical Company, 2001). When the NTS biosensor chips were tested with *Salmonella*,

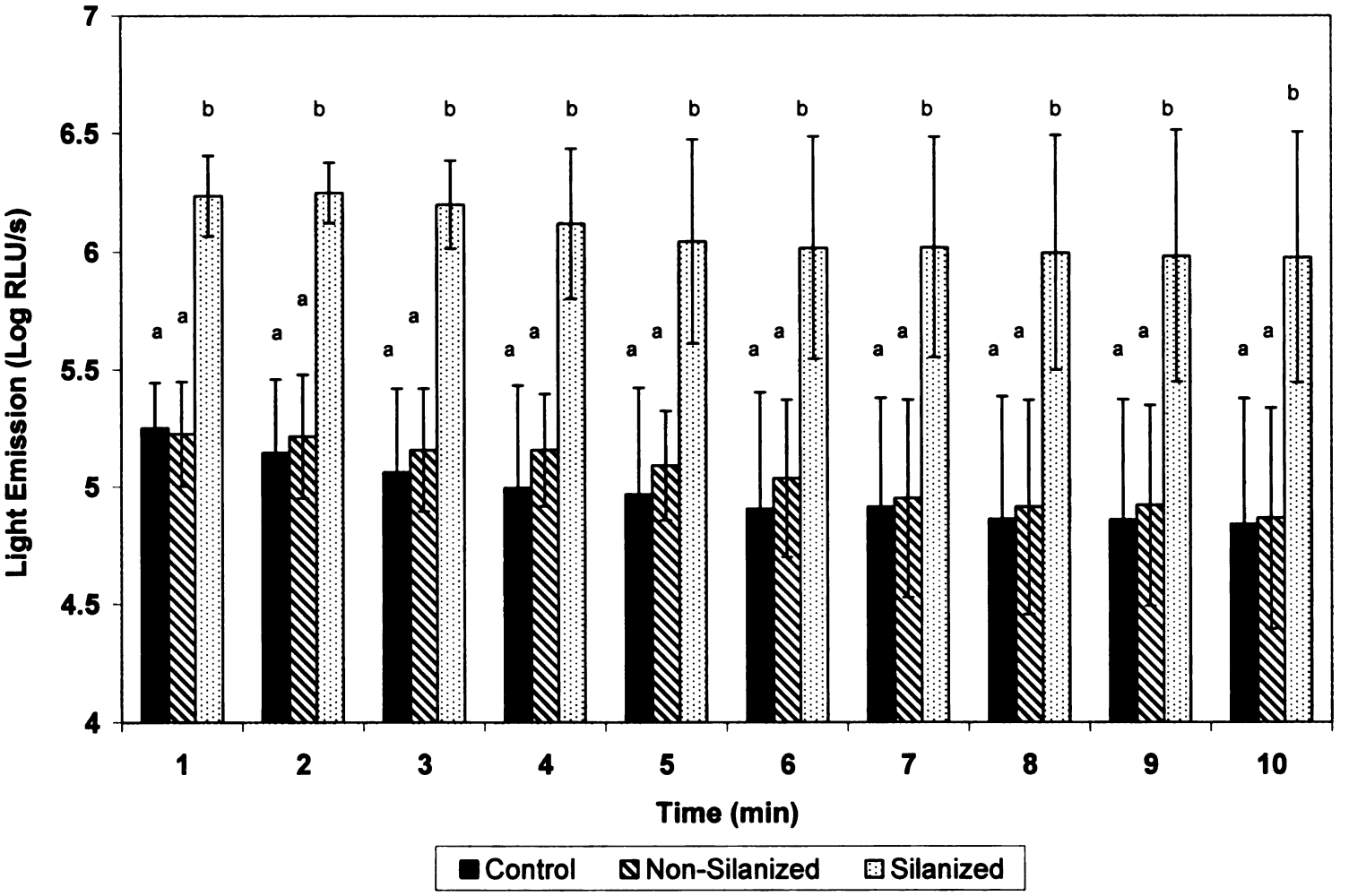

Figure 5-19. Comparison of light emission for *Salmonella* pure culture with silanized and non-silanized nano-tubular Si biosensor against silanized control. Different superscript over the bars indicates significantly different light emission ($p<0.05$).

light was measured for only 10 min, because significant difference in light emission could already be observed after the first minute in silanized chips with *Salmonella* when compared to the control. Light readings were measured to test the hypothesis that silanization of NTS was an essential step in functionalization of the biosensor chip, accomplished by comparing the kinetics of *Salmonella* on non-silanized chips against that of silanized chips. Light emission from a non-silanized chip comparable to that of a silanized chip would indicate successful functionalization by physical adsorption of antibodies directly onto the NTS surface or through entrapment within the nano-tubes. However, no significant difference ($p>0.05$) in light emission of the non-silanized chip (with *Salmonella*) was observed when compared to the silanized and non-silanized control (no *Salmonella*), indicating that physical adsorption or entrapment of antibodies was not a reliable method of antibody immobilization. The physical adsorption process used for antibody binding explains the lower light emission of the non-silanized NTS biosensor chip when compared to that of the silanized chip. No (or significantly lower) binding of the antibody resulted from physical adsorption due to lack of a stable bond/link between the NTS surface and the antibody.

Silanization of NTS enables formation of a stable covalent bond between the cross linker and the silicon surface, which in turn binds the antibody to the surface (Bhatia *et al.*, 1989). When the NTS surface is not silanized, antibodies are not covalently bound to the surface. Physically entrapped antibodies in the non-silanized biosensor chip could easily get removed during any of the blocking and washing steps used in the optical detection method, thus explaining the lower light emission of the chip. As observed in the current study, other investigators have successfully employed silanization for

immobilization of biological molecules on silica (Bhatia *et al.*, 1989) and SiO_2-TiO_2 optical wave-guide (Trummer *et al.*, 2001) surfaces.

Functionalization of the NTS into an immuno-biosensor was achieved using silanization and immobilization of antibodies as confirmed by the chemiluminescence-based optical detection method. Comparison of light emission from silanized and non-silanized biosensor chips proved silanization to be an essential step in the functionalization of the biosensor. Light emission of the NTS biosensor chip was not significantly higher than that of the planar Si chip, indicating limited advantage of NTS as an immuno-biosensor platform when used for bacterial detection. Application of the NTS biosensor to virus and antibody detection is yet to be investigated.

5.3.4 Functionalization of the Nano-Tubular Si Platform with *Salmonella* DNA Probes into a Nucleic Acid-Based Biosensor

5.3.4.1 Polymerase chain reaction

A clinical strain of *Salmonella* Enteritidis (strain S-64, MDCH collection) was used as the target for development of the nucleic acid-based biosensor. The chosen PCR primers and the DNA capture probe were from the insertion (*iel*) gene of *Salmonella* Enteritidis with a very high degree of specificity for *Salmonella* species (E value of 2.0 x 10^{-5} for *Salmonella* versus 5.1 for the closest non *Salmonella* target with the capture probe). PCR was successfully performed with overnight cultures of *S.* Enteritidis grown in TSBYE as confirmed by gel electrophoresis (Figure 5-20).

The PCR amplified DNA product should be 505 bp long (positions 542 to 1047 of the *iel* gene of *Salmonella* Enteritidis). Figure 5-20 shows that the PCR product (Lanes S1 and S2) corresponds to the 500 bp DNA band of the marker (Lane M), confirming the

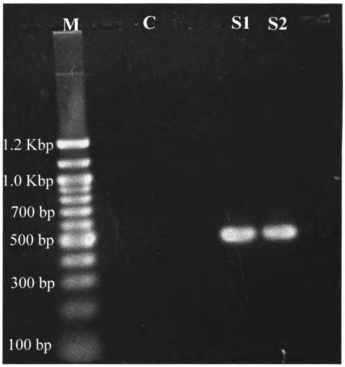

Figure 5-20. Gel electrophoresis of PCR-amplified *Salmonella*: S1 and S2 - 5 µl culture used for PCR. *Key: M – Marker, C – negative control, S – Salmonella*

length of the PCR product. Following PCR and gel electrophoresis confirmation, the concentration and purity of DNA in the PCR amplified (pooled) sample were determined using spectrophotometer measurements. The DNA concentration in the PCR amplified (S1 and S2 pooled together) sample was 132.61 µg/ml with an $A_{260/280}$ ratio of 1.99, indicative of the high quality of the PCR DNA product.

5.3.4.2 AFM characterization of nano-tubular Si DNA biosensor

The NTS platform was functionalized into a DNA-based biosensor by immobilizing the DNA capture probe specific to *S.* Enteritidis. The above PCR amplified DNA product was diluted appropriately to obtain a 1 ng/µl solution of DNA and allowed to hybridize to the capture probe immobilized on the NTS DNA biosensor. The process of functionalization and target DNA hybridization was confirmed using atomic force microscopy (AFM) images. Figures 5-21 to 5-23 show the surface profiles of NTS DNA

biosensor chips prior to DNA probe immobilization, after DNA probe immobilization, and following target DNA hybridization at the nanometer scale. The profile of the NTS surface prior to DNA probe immobilization (immediately after silanization) confirmed the SEM image characterization of NTS with pores distributed evenly throughout the surface (Figure 5-21). After probe immobilization, a small change in the surface profile was observed, with some surface leveling and deposition of matter in the pores and inter-pore spaces occurring on the chip surface (Figure 5-22). Following target DNA hybridization on the NTS biosensor chip (and after washing steps and drying under nitrogen gas stream), AFM showed that the surface profile changed drastically with smoothening of the surface observed at the nanometer scale, indicating extensive deposition of matter in the pores as well as on the inter-pore spaces (Figure 5-23). This is hypothesized to be from the hybridization of DNA target to the biosensor chip surface, as care was taken to prevent non-specific binding of organic matter to the biosensor surface through use of surface blocking agents (bovine serum albumin), washing steps, and a high degree of hybridization specificity by maintaining the hybridization temperature at 59°C (melting point of capture probe was 60°C) with a standard hybridization buffer.

5.3.4.3 Electrochemical detection of DNA using nano-tubular Si biosensor

A cyclic voltammogram (CV) is a valuable and convenient tool to monitor the barrier of the modified electrode because electron transfer between the solution species and the electrode must occur by tunneling either through the barrier or through the defects in the barrier. When an electrode surface is modified by some materials, the electron-transfer kinetics of $Fe(CN)^{-4}_6$ is perturbed, in turn affecting the output current. Therefore, cyclic voltammetry was chosen as a method to investigate the changes

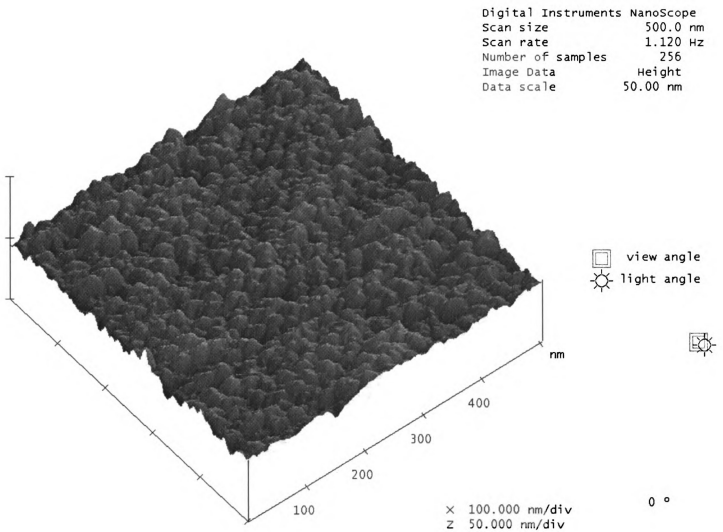

Figure 5-21. AFM image of NTS-based DNA biosensor chip prior to DNA probe immobilization.

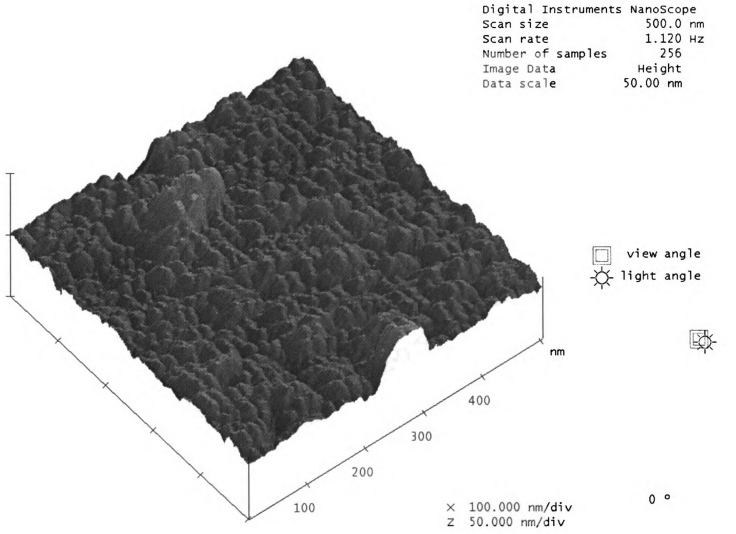

Figure 5-22. AFM image of NTS-based DNA biosensor chip after DNA probe immobilization.

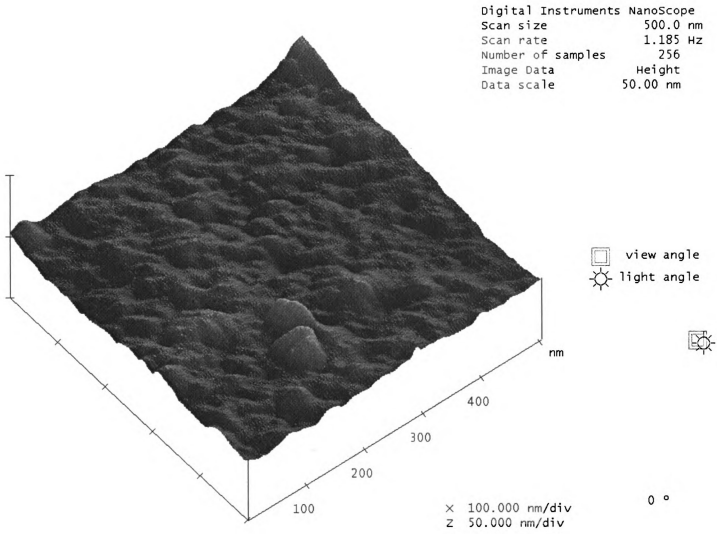

Figure 5-23. AFM image of NTS-based DNA biosensor chip after target DNA hybridization.

in electrochemical behavior of the system after each step, from silanization to DNA target hybridization.

Figure 5-24 illustrates the CV obtained with the NTS biosensor chip using 5 mM potassium ferrocyanide as the redox couple marker in 1 M potassium nitrate after different processing steps. Unlike the typical CV (Section 2.4.4 above, Figure 2-5 on page 54), the CV obtained with the NTS biosensor did not have a characteristic cathodic (I_{pc}) or anodic peak current (I_{pa}), thus showing signs of irreversibility of reactions. The current went to a steady-state maximum on the cathodic side and was not peak-shaped (Figure 5-24).

When the rate of electron transfer is sufficiently slow so that the potential no longer reflects the equilibrium activity of the redox couple ($Fe(CN)_6^{3-} + e^- \leftrightarrow Fe(CN)_6^{4-}$) at the working electrode surface, the reaction is considered irreversible. In such a case, the potentials corresponding to the cathodic and anodic current peaks (E_p) would change as a function of the scan rate. This steady-state current could be explained by envisioning the working electrode (NTS biosensor) placed at the bottom of the 3-electrode electrochemical cell (Chapter 4, Figure 4-7 on page 111) as a "dot", with the diffusion boundary layer being hemispherical in shape extending up into the solution. The amount of ferrocene diffusing to the working electrode surface would be defined by the volume enclosed by the hemisphere, which is much smaller than a plane projecting into the solution as in the case of a planar electrode immersed completely into the electrochemical cell (Wang, 2000). The atypical CV response could thus be explained by the limited area available for the diffusion layer at the bottom of the cell for electron transfer combined with the use of silicon as the working electrode (lower conductivity) instead of platinum

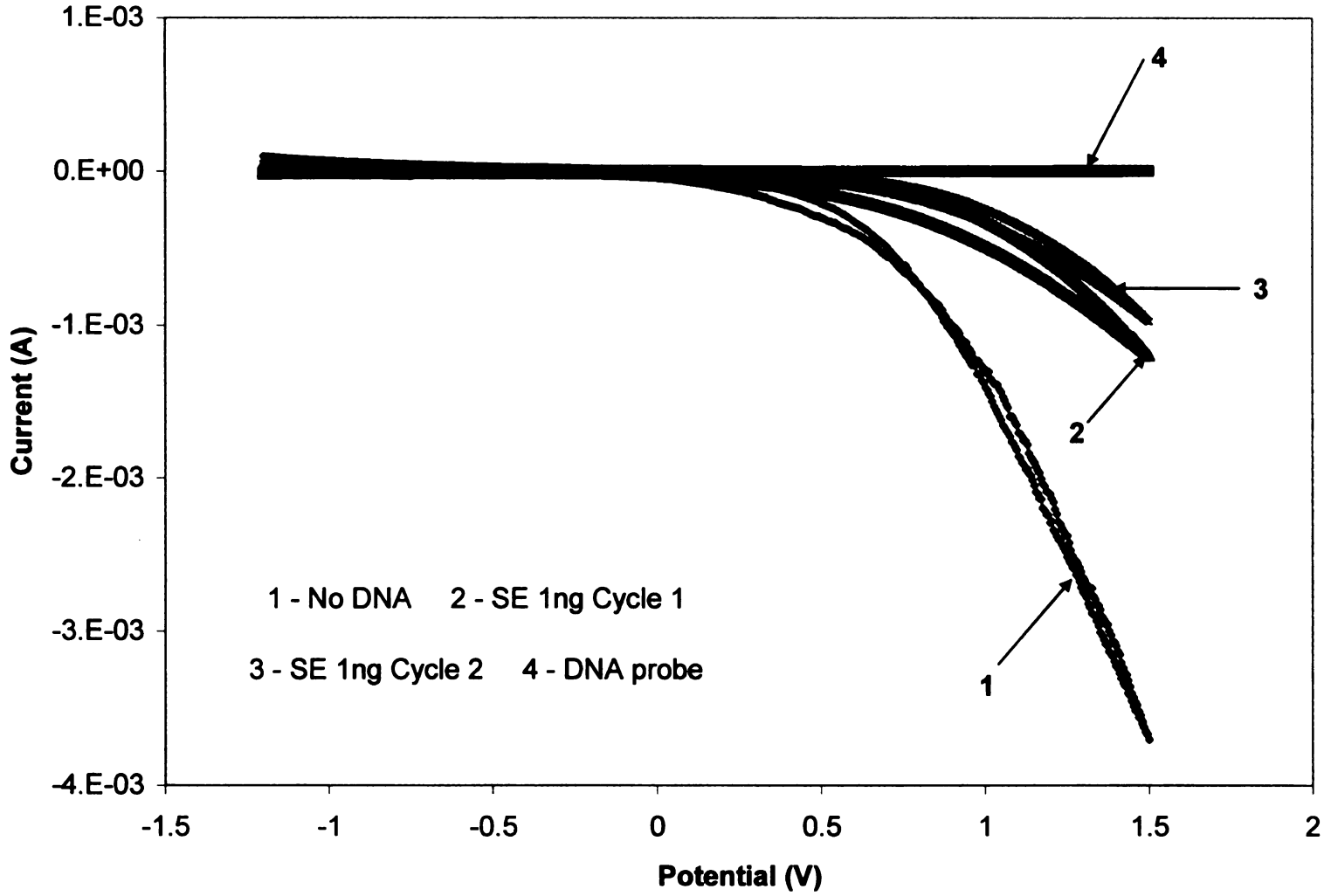

Figure 5-24. Cyclic voltammogram for a pure culture of *Salmonella* Enteritidis obtained with NTS-based DNA biosensor chip.

that is used to generate typical CV curves. Irreversibility of the reaction could also be observed from the CV profiles for the two consecutive cycles (Lines 2 and 3 in Figure 5-24). There was a consistent reduction (decay) in the current output on the anodic side from cycle 1 to cycle 2 for each set of hybridization conditions at various DNA concentrations (data shown in Sections 4 and 5). Running more than two CV cycles reduced the current output even further (data not shown).

Without any DNA deposited on the NTS surface, the resistance or barrier offered to the flow of electrons was the least, resulting in a CV with a high current output (Line 1 in Figure 5-24). After immobilization of the single-stranded DNA capture probe, the current drastically decreased on the anodic side (Line 4 in Figure 5-24). After hybridization of the DNA target (1 ng/μl), the obtained CV showed relatively higher current values on the anodic side (Lines 2 and 3 in Figure 5-24) when compared to the chip with the immobilized DNA probe only.

Immobilization of the single-stranded DNA would form an insulating diffusion barrier on the working electrode surface, hindering electron transfer through the working electrode. This insulating phenomenon was similar to that observed in previous studies that employed physical parameters (besides the conductivity of the working electrode or reporting molecules employed) for direct label-free electrochemical detection of specific DNA sequences. For example, Souteyrand *et al.* (1997) used a probe-coated field-effect silicon device for in situ impedance measurements of DNA sequences. The device displayed well-defined shifts of the impedance curves, corresponding to changes in surface charge induced by base-pair recognition. Similarly, Berggren *et al.* (1999) demonstrated a positive change in the capacitance of a thiolated (insulating)

oligonucleotide modified gold electrode caused by hybridization of the complementary DNA strand (and the corresponding displacement of solvent molecules from the surface). An even greater insulating property of a single-stranded DNA monolayer on the gold electrode was observed in a previous study by the same authors (Berggren and Johansson, 1997).

The intrinsic electroactivity of DNA (Palecek, 1996) restores the anodic current upon hybridization of the target DNA. Of the four nucleobases, guanine and adenine are readily oxidized. The decreased guanine and adenine response of the immobilized oligonucleotide probe restored upon hybridization of the complementary strand has been used for detecting RNA hybridization (Wang *et al.*, 1995). Other studies have also demonstrated a similar increase in signal with hybridization of the target DNA strand with and without reporting molecules such as Hoechst 33258 or $[Ru(bpy)_3]^{3+}$ (Wang, 1999; Kobayashi *et al.*, 2004; Lee *et al.*, 2004; Li and Hu, 2004).

5.3.4.4 Statistical analysis of CV data

As a typical CV curve with anodic and cathodic peak currents could not be obtained with the NTS biosensor chip, the usual statistical method of peak analysis could not be employed to determine significant differences between mean current outputs before and after target DNA hybridization. The alternate procedures described in Chapter 4:Section 4.4.4 were employed to determine the performance of the NTS and planar Si biosensor chips in terms of sensitivity and specificity. These are described in greater detail in the results and discussion for Objectives 4 and 5.

5.4 OBJECTIVE 4

Determination of sensitivity of the nano-tubular silicon DNA biosensor in pure culture.

5.4.1 Sensitivity of the Biosensor using PCR amplified DNA

PCR-amplified *Salmonella* DNA from Objective 3 (Section 5.3.4.1 above) was serially diluted so that the final concentration ranged from 10^{-9} μg/μl to 10^{-15} μg/μl. Following NTS and planar Si DNA biosensor functionalization and pre-hybridization cyclic voltammetry measurements, each of these DNA concentrations was tested with the two Si-based biosensors.

The specific DNA concentration to be tested with the biosensor was diluted to a total volume of 5 ml using the DNA hybridization buffer and then heated to 59°C. The chips to be tested were then placed in the hybridization buffer solution containing the target DNA and allowed to hybridize for 45 min. Preliminary testing with a hybridization time of 60 min versus 45 min showed no added advantage in terms of signal output. Thus, a hybridization time of 45 min was chosen for all experiments giving a total assay time of 60 min (45 min for hybridization and 15 min for the cyclic voltammetry testing procedure). After hybridization, the NTS and planar Si chips were subjected to cyclic voltammetry for the final time.

5.4.1.1 Cyclic voltammograms

Figure 5-25 shows the CV curves for the NTS biosensor chips tested for different PCR-amplified *Salmonella* DNA concentrations with the mean current values plotted against the corresponding applied potential. For the sake of clarity of the cyclic voltammograms (CVs) in Figure 5-25, the standard errors of the curves are not shown.

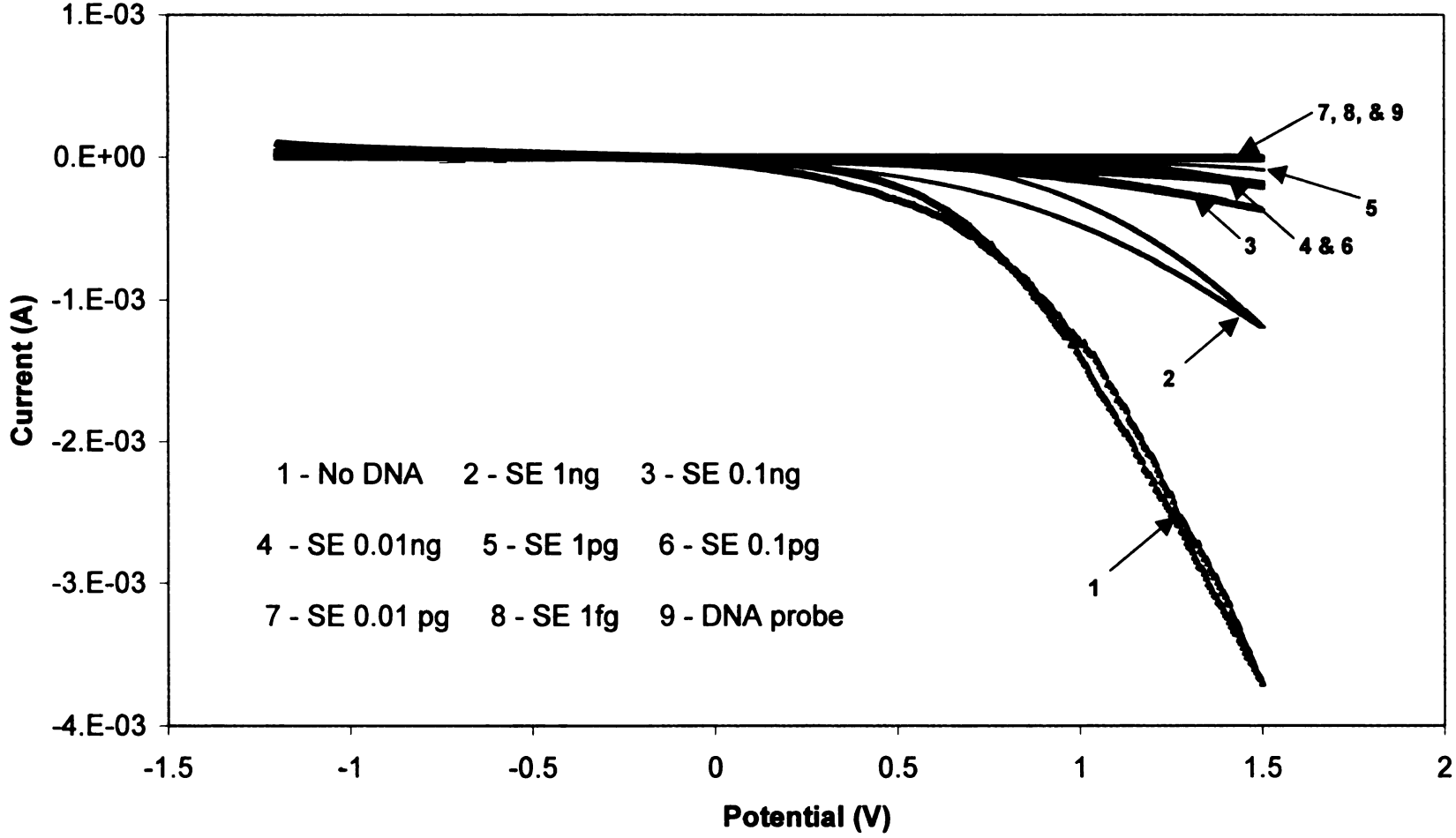

Figure 5-25. Cyclic voltammograms for serially diluted PCR-amplified *Salmonella* Enteritidis DNA obtained with NTS-based DNA biosensor (sensitivity testing).

Moreover, for ease of comparison, the CVs for 1 ng/μl biosensor chips obtained prior to DNA immobilization (Line 1 – No DNA) and after DNA probe immobilization (Line 9 – DNA probe) are the only ones included along with the CVs for each DNA concentration after the target DNA hybridization. The CVs obtained for pure culture DNA with NTS as well as those for PCR-amplified and pure culture DNA with planar Si will follow the same pattern when presented in the following sections.

The anodic current for the CV before DNA probe immobilization (Line 1) was the highest of all the samples tested with the NTS biosensor platform. The output current cycled between 10 μA on the cathodic side to about -4 mA on the anodic side. The CV for the NTS biosensor chip after DNA probe immobilization (Line 9) was significantly lower (as determined statistically later in Section 5.4.1.2) than the CV prior to probe immobilization with the current output cycling from 1 μA to –1 μA. After the target DNA hybridization, anodic current increased again as observed previously (Section 5.3.4.3). The extent of change in the anodic current from that of the DNA probe chip was related to the amount of target DNA getting hybridized to the biosensor chip, with the highest change in anodic current observed for 1 ng/μl DNA concentration and the lowest change observed for 0.01 pg/μl and 1 fg/μl (Note: Purified DNA yield from an overnight *Salmonella* Enteritidis culture was 10 ng/μl).

When planar Si biosensor chips were tested with different PCR-amplified *Salmonella* DNA concentrations, the anodic current for the CV before DNA probe immobilization (Line 1 – No DNA) was two orders of magnitude lower than that obtained with NTS biosensor chip (Figure 5-26). The output current cycled between 1 μA on the cathodic side to about -10 μA on the anodic side. The CV for the planar Si

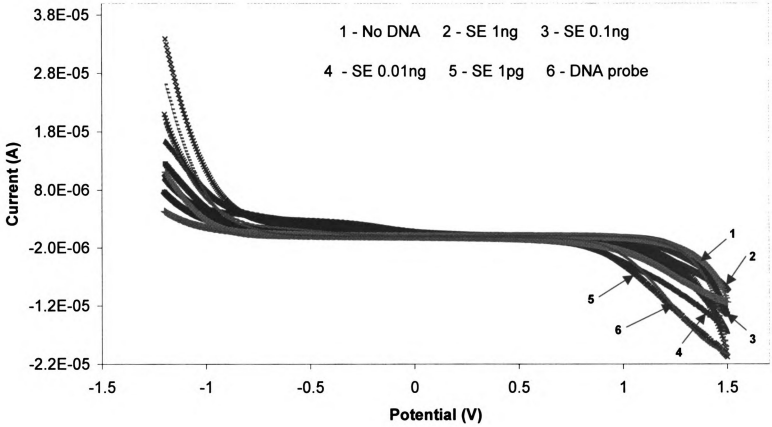

Figure 5-26. Cyclic voltammograms for serially diluted PCR-amplified *Salmonella* Enteritidis DNA obtained with planar Si-based DNA biosensor chip (sensitivity testing).

biosensor chip after DNA probe immobilization (Line 9) and that after the target DNA hybridization were all in the same range, with the current output cycling from about 4 μA to -22 μA. In contrast to the CVs obtained for the NTS biosensor chips, no specific patterns of interest could be observed for the planar Si chips. Based Figure 5-25 on and Figure 5-26, there appears to be a significant difference in the performance of the sensor chips (to be confirmed by statistical analysis). The significantly higher surface area with NTS biosensor chips compared to planar Si chips would provide greater area for the DNA hybridization and subsequent electrochemical reaction kinetics to take place, thus explaining the observed difference in performance trends.

5.4.1.2 Data analysis

5.4.1.2.1 Modeling and curve smoothening

The data analysis approach used in this research objective was to use the maximum amount of information available from the CVs (versus usual peak analysis). Non-linear modeling could use all or most of the data points generated by the cyclic voltammetry. However, the atypical CVs obtained with NTS and planar Si biosensor chips made it difficult to fit one common model to the data collected under different experimental conditions. Therefore, a generalized additive model was used to model the current as a function of voltage. This model would provide a basis for comparison of the different curves (Blank – no DNA, Before – before DNA target hybridization, and After – after DNA target hybridization) for each experimental condition. However, modeling based on curve smoothening could not be used as concrete statistical evidence of significant mean differences due to the general assumptions underlying the smoothening *SPLINE* process in SAS®. Splines are curves, which are usually required to be continuous and smooth,

defined as piecewise polynomials of degree *n* with function values and first *n*-1 derivatives that agree at the points where they join. The abscissa values of the join points are called knots. Splines with no knots are generally smoother than splines with knots, Splines with few knots are generally smoother than splines with many knots; however, knots give the curve freedom to bend to more closely follow the data (SAS Online Doc, 2000). While splines provide a very close fit to all the data points, the model has vastly different polynomial functions for different experimental conditions, and thus a direct comparison is not feasible. Figure 5-27 and Figure 5-28 show a sample of the smoothened curves obtained from SAS using the SPLINE model for 1 ng/μl and 0.01 ng/μl of PCR-amplified DNA with the NTS biosensor chip. While the SPLINE model was helpful in smoothening the CV curves with a very high variability (especially planar Si chips) from replicate to replicate, it also reduced the variability of the data. Hence, the 95% confidence interval determined by the SPLINE model was calculated based on the smoothened curve data values (with very low sample variance), leading to the narrow bands for the confidence interval. These smoothened 95% bands could not be used to determine the probability of two curves being significantly different from each other, and thus were not used hereafter to compare the CV curves.

5.4.1.2.2 Analysis of Delta Q values

Of the three possible data analysis approaches that were considered, analysis of Delta Q values was the best methodology. Delta Q is the integral of current across the selected set of points with respect to time. Thus, ΔQ takes into account all the data points in the curve in order to generate one representative cumulative charge value for each CV curve. This methodology was better than the peak analysis procedure because it considered all

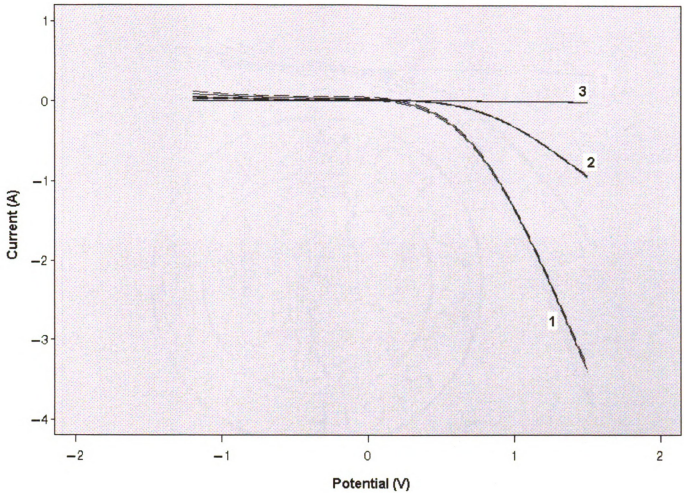

Figure 5-27. Spline curve for PCR-amplified *Salmonella* Enteritidis DNA on nano-tubular Si biosensor at 1ng/µl concentration level. *Key: 1 – No DNA, 2 – After DNA Hybridization, and 3 – Before DNA Hybridization.*

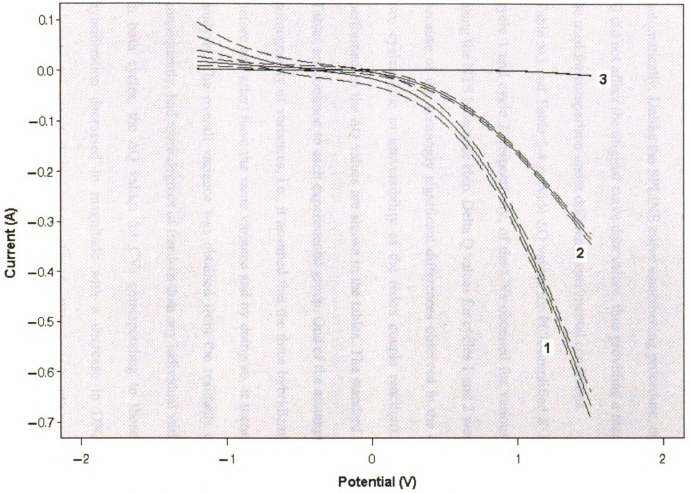

Figure 5-28. Spline curve for PCR-amplified *Salmonella* Enteritidis DNA with nano-tubular Si biosensor at 0.1 ng/µl concentration level. *Key: 1 – No DNA, 2 – After DNA Hybridization, and 3 – Before DNA Hybridization.*

the points in the curve as opposed to a single peak point (anodic and/or cathodic current). Moreover, when calculating the integral charge value, the time factor was also used along with the voltage, incorporating any effects of the time factor into the model automatically. Unlike the SPLINE curve smoothening procedure, calculation of the Delta Q did not affect the original curve data values, thus providing a measure that could easily be used for comparison under different experimental conditions.

Table 5-3 and Table 5-4 provide ΔQ values for PCR-amplified *S.* Enteritidis DNA from cycle 1 and cycle 2, respectively, of the CVs obtained for various DNA concentrations using the NTS biosensor chip. Delta Q values for cycles 1 and 2 were analyzed separately because of the seemingly significant differences observed in the current output for the two cycles (due to irreversibility of the redox couple reaction). Results of ANOVA performed on the ΔQ values are shown in the tables. The standard errors provided in the Tables are common to each experimental group. One of the assumptions of ANOVA was homogeneity of variances, i.e., it assumed that the three hybridization treatments (blank, before and after) have the same variance and by doing so, it increased the power of the analysis. The overall variance was obtained from the residuals of all treatments, and consequently, had more degrees of freedom than any individual variance.

In both cycles, the ΔQ values for CVs corresponding to those 'After' DNA target hybridization decreased in magnitude with a decrease in DNA concentration. For example, for cycle 1, the mean ΔQ values for CVs corresponding to 1 ng, 0.1 ng and 0.01 ng were -45.767±3.72, -14.467±2.33, and -12.633±1.79 mC, respectively. This trend was observed in Cycle 2 as well. Delta Q values corresponding to CVs 'Before' DNA hybridization remained fairly steady at -18 to -57 μC irrespective of the concentration of

Table 5-3. Integral charge (ΔQ, milli coulombs) values for PCR-amplified *S. Enteritidis* DNA with NTS biosensor at various concentrations using cycle 1 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-173.333±3.72^{c}	-0.231±3.72^{a}	-45.767±3.72^{b}
0.1 ng	-60.767±2.33^{c}	-0.306±2.33^{a}	-14.467±2.33^{b}
0.01 ng	-29.867±1.79^{c}	-0.237±1.79^{a}	-12.633±1.79^{b}
1 pg	-77.167±19.25^{a}	-0.183±19.25^{a}	-7.773±19.25^{a}
0.1 pg	-23.600±2.73^{b}	-0.576±2.73^{a}	-5.933±2.73^{a}
0.01 pg	-48.400±5.40^{b}	-0.571±5.40^{a}	-0.076±5.40^{a}
1 fg	-15.867±1.22^{b}	-0.461±1.22^{a}	-0.030±1.22^{a}

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Table 5-4. Integral charge (ΔQ, milli coulombs) values for PCR-amplified *S. Enteritidis* DNA with NTS biosensor at various concentrations using cycle 2 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-148.000±2.70^{c}	-0.202±2.70^{a}	-39.667±2.70^{b}
0.1 ng	-56.833±1.24^{c}	-0.249±1.24^{a}	-13.733±1.24^{b}
0.01 ng	-25.000±2.49^{b}	-0.174±2.49^{a}	-9.797±2.49^{a}
1 pg	-51.100±1.15^{c}	-0.153±1.15^{a}	-6.267±1.15^{b}
0.1 pg	-24.133±1.94^{b}	-0.210±1.94^{a}	-4.770±1.94^{a}
0.01 pg	-49.000±6.17^{b}	-0.349±6.17^{a}	-0.066±6.17^{a}
1 fg	-14.267±1.55^{b}	-0.192±1.55^{a}	-0.040±1.55^{a}

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

target DNA hybridized later (Table 5-3). 'Blank' ΔQ values prior to DNA immobilization on the NTS biosensor chips varied the most from about -16 to -173 mC for various DNA concentrations. However, this variation did not affect the biosensor performance as the focus of the assay was the mean difference between ΔQ values for the 'Before' and the 'After' target DNA hybridization steps. The NTS biosensor chips could detect PCR-amplified *S.* Enteritidis DNA in the dynamic detection range of 1 ng to 10 pg with cycle 1, and 1 ng to 1 pg with cycle 2. Thus, the lower limit of detection (sensitivity) for NTS biosensor chips with PCR-amplified *Salmonella* DNA was between 1 pg and 10 pg. This difference in the dynamic range of detection and sensitivity could be due to the larger variation in ΔQ values observed during cycle 1 as compared to cycle 2 under all experimental conditions investigated. The sensitivity for NTS was similar to that established in previous studies using modified gold surfaces as the biosensor platform in combination with various reporting molecules (Wang *et al.*, 1996; Li and Hu, 2004; Fu *et al.*, 2005).

Sensitivity analysis of the planar Si showed that the biosensor chip was unable to successfully detect the PCR-amplified DNA target even at 1 ng level (

Table 5-5). With both the cycles, there was no significant difference between the "Before" and "After" hybridization steps (Table 5-6) at any DNA concentration level. The low current CV outputs for planar Si biosensor chips (resulting from significantly lower surface area available for the electrochemical reaction) makes planar Si more susceptible to variations in the data from replicate to replicate (biological or technical).

Table 5-5. Integral charge (ΔQ, milli coulombs) values for PCR-amplified *S. Enteritidis* DNA with planar Si biosensor at various concentrations using cycle 1 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-0.197±0.13[a]	0.132±0.13[a]	-0.014±0.13[a]
0.1 ng	-0.167±0.11[a]	-0.037±0.11[a]	-0.027±0.11[a]
0.01 ng	0.094±0.10[a]	0.090±0.10[a]	-0.021±0.10[a]
1 pg	0.038±0.11[a]	0.022±0.11[a]	0.069±0.11[a]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Table 5-6. Integral charge (ΔQ, milli coulombs) values for PCR-amplified *S. Enteritidis* DNA with planar Si biosensor at various concentrations using cycle 2 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-0.041±0.12[a]	0.174±0.12[a]	-0.018±0.12[a]
0.1 ng	-0.005±0.08[a]	0.056±0.08[a]	0.011±0.08[a]
0.01 ng	0.012±0.05[a]	0.051±0.05[a]	-0.003±0.05[a]
1 pg	0.034±0.08[a]	0.079±0.08[a]	0.004±0.08[a]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

5.4.2 Sensitivity of the Biosensor Using DNA from Pure Culture

The DNA concentration in the overnight *Salmonella* pure culture was 113.85 μg/ml with an $A_{260/280}$ ratio of 2.34, indicative of very high quality DNA. This stock solution was appropriately diluted for sensitivity testing with NTS and planar Si biosensors.

5.4.2.1 Cyclic voltammograms

Figure 5-29 and Figure 5-30 show the CV curves for the NTS biosensor and planar Si chips, respectively, tested with different pure culture (extracted) *Salmonella* DNA concentrations obtained by plotting mean current values against the corresponding applied potential. As with CVs for PCR-amplified DNA, standard errors for the curves are not shown. The CVs obtained for pure culture DNA with NTS as well as with planar Si followed the same pattern as observed with CV output for PCR-amplified DNA.

The anodic current for the CV before DNA probe immobilization (Line 1) was the highest of all the samples tested with the NTS biosensor platform. The output current cycled between 10 μA on the cathodic side to about -1 mA (slightly lower than that for PCR-amplified DNA) on the anodic side. The CV for the NTS biosensor chip after DNA probe immobilization (Line 9) was significantly lower (as determined statistically through delta Q analysis in Section 4.2.2) than the CV prior to probe immobilization with the current output cycling from 1 μA to –1 μA. After the target DNA hybridization, anodic current increased again as observed previously, with the increase in magnitude of the anodic current being the highest for 0.01 ng/μl DNA. The other three concentrations also showed a change over the anodic current from the CV for the DNA probe.

When planar Si biosensor chips were tested, the anodic current for the CV before DNA probe immobilization (Line 1 – No DNA) was again lower than that obtained with NTS biosensor chip (Figure 5-30). The output current cycled between 25 μA on the cathodic side to about -20 μA on the anodic side. The CV for the planar Si biosensor chip after DNA probe immobilization (Line 9) and that after the target DNA hybridization were all in the same range. Based on Figure 5-29 and Figure 5-30, there appears to be a

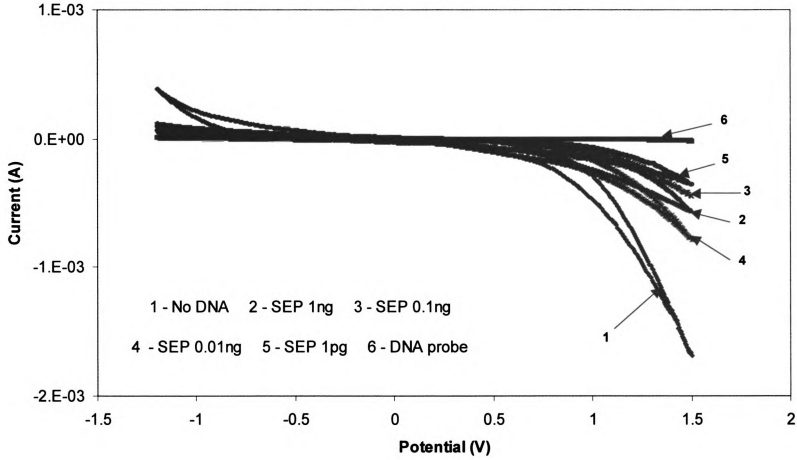

Figure 5-29. Cyclic voltammograms for serially diluted (extracted) pure culture *Salmonella* Enteritidis DNA obtained with NTS-based DNA biosensor chip (sensitivity testing).

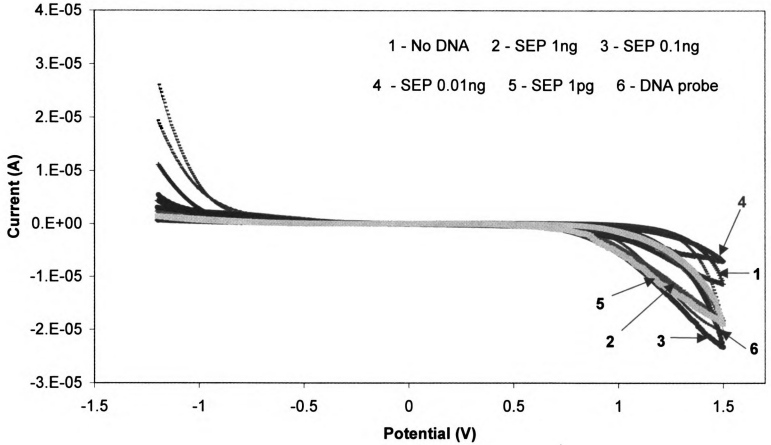

Figure 5-30. Cyclic voltammograms for serially diluted (extracted) pure culture *Salmonella* Enteritidis DNA obtained with planar Si-based DNA biosensor chip (sensitivity testing).

significant difference in the performance of the sensor chips (also to be confirmed by statistical analysis using interactive effects contrast for platform).

5.4.2.2 Analysis of Delta Q values

Delta Q values for CVs of pure culture *S.* Enteritidis DNA (cycle 1 and cycle 2, respectively) at various DNA concentrations using the NTS biosensor chip are presented in Table 5-7 and Table 5-8. Delta Q values for cycles 1 and 2 were analyzed separately as before. Results of ANOVA performed on the ΔQ values are shown in the tables. 'Blank' ΔQ values prior to DNA immobilization on the NTS biosensor chips varied more in cycle 1 than cycle 2 (Table 5-7 and Table 5-8). In both cycles, the ΔQ values for CVs corresponding to those 'After' DNA target hybridization increased in magnitude compared to that 'Before' target hybridization. For example, for cycle 1, the mean ΔQ values for CVs corresponding to 1 ng and 0.01 ng – 'Before' were -0.028 ± 0.81, and 0.008 ± 4.63 mC, while 'After' were -75.700 ± 0.81, and -26.633 ± 4.63 mC, respectively. This trend was observed in Cycle 2 as well. The values of ΔQ corresponding to CVs 'Before' and 'After' DNA hybridization varied more than that of PCR-amplified DNA (Table 5-7).

The NTS biosensor chips could detect extracted pure culture *S.* Enteritidis DNA in the dynamic detection range of 1 ng to 0.01 ng for both cycles. Thus, the sensitivity for NTS biosensor chips with *Salmonella* DNA was 0.01 ng. This difference in the dynamic range of detection and sensitivity between PCR amplified and pure-culture DNA could be due to the non-uniform DNA product (in terms of strand length) hybridizing to the DNA probe immobilized on the surface. Since the presence of guanine and adenine moieties in double-stranded DNA increases the anodic current, possible hybridization of spatially

Table 5-7. Integral charge (ΔQ, milli coulombs) values for pure culture *S.* Enteritidis (extracted) DNA with NTS biosensor at various concentrations using cycle 1 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-30.633±0.81[b]	-0.028±0.81[a]	-75.700±0.81[c]
0.1 ng	-66.733±0.66[c]	0.339±0.66[a]	-10.113±0.66[b]
0.01 ng	-29.500±4.63[b]	0.008±4.63[a]	-26.633±4.63[b]
1 pg	-105.133±56.41[a]	-0.947±56.41[a]	-25.877±56.41[a]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Table 5-8. Integral charge (ΔQ, milli coulombs) values for pure culture *S.* Enteritidis (extracted) DNA with NTS biosensor at various concentrations using cycle 2 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	-45.133±0.99[c]	-0.030±0.99[a]	-28.333±0.99[b]
0.1 ng	-62.267±0.68[c]	0.329±0.68[a]	-9.630±0.68[b]
0.01 ng	-24.333±4.16[b]	0.019±4.16[a]	-18.300±4.16[b]
1 pg	-68.800±53.87[a]	-0.918±53.87[a]	-15.060±53.87[a]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

adjacent strands to each other (after hybridization of the small fragment of the *iel* gene to the capture probe) could lead to increased current output. Table 5-7 and Table 5-8 show no particular trend for the increase in ΔQ values for CVs corresponding to 'After' hybridization, possibly resulting from the random hybridization of *Salmonella* genomic

DNA to each other. The variability in ΔQ values is also much higher for pure culture *Salmonella* DNA as compared to PCR-amplified *Salmonella* DNA that are very uniform in size. Hence, the sensitivity for NTS with pure culture DNA was one order of magnitude lower than PCR-amplified DNA.

Sensitivity analysis of the planar Si showed that the biosensor chip was unable to successfully detect the pure culture DNA target even at 1 ng level (Table 5-9 and Table 5-10). With both the cycles, there was no significant difference between the "Before" and "After" hybridization steps (Table 5-9) at any DNA concentration level. However, a few instances were observed where the ΔQ values for 'Blank' were significantly different from that of 'After'. For example, ΔQ values for cycle 1 with 1 ng DNA concentration were -0.085±0.09, -0.088±0.09, and -0.414±0.09 mC, respectively, for 'Blank', 'Before' and 'After' states of DNA hybridization, wherein the 'Blank' was significantly different from the 'After' ($p<0.05$), but the mean difference between 'After' and 'Before' was non-significant ($p>0.05$). Again, the low current CV outputs for planar Si biosensor chips made planar Si more susceptible to variations in the data and unable to detect the DNA hybridization event at any of the levels tested.

5.4.2.3 Interactive effects of variables

Investigation of Single or Main effects using factorial ANOVA analyses provided some insight into possible interactive effects of variables on the output of the NTS and planar Si platforms. Four-way interaction of DNA hybridization event, DNA source, DNA concentration and the platform was shown to be highly significant ($p<0.01$).

Further investigation of the Single effect of platform showed NTS to have a highly significant effect on DNA hybridization ($p<0.0001$), as did the DNA source (PCR or

pure) ($p<0.05$). No interactive effects between DNA source and platform were found to be significant ($p=0.70$). Complete SAS data analysis output is provided in the Appendix C: Section C.2.

Table 5-9. Integral charge (ΔQ, milli coulombs) values for pure culture *S. Enteritidis* (extracted) DNA with planar Si biosensor at various concentrations using cycle 1 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	0.085±0.09[a]	-0.088±0.09[ab]	-0.414±0.09[b]
0.1 ng	-0.307±0.16[a]	0.080±0.16[a]	-0.330±0.16[a]
0.01 ng	-0.298±0.12[a]	-0.081±0.12[a]	-0.212±0.12[a]
1 pg	-0.113±0.11[a]	-0.117±0.11[a]	-0.502±0.11[a]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Table 5-10. Integral charge (ΔQ, milli coulombs) values for pure culture *S. Enteritidis* (extracted) DNA with planar Si biosensor at various concentrations using cycle 2 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
1 ng	0.073±0.05[a]	-0.083±0.05[ba]	-0.236±0.05[b]
0.1 ng	-0.131±0.08[a]	-0.056±0.08[a]	-0.377±0.08[a]
0.01 ng	-0.057±0.03[a]	-0.014±0.03[a]	-0.095±0.03[a]
1 pg	0.017±0.04[a]	-0.034±0.04[ba]	-0.170±0.04[b]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

5.5 OBJECTIVE 5

Determination of specificity of the nano-tubular silicon biosensor using mixed bacterial cultures.

5.5.1 Specificity Testing

In order to determine the specificity of the biosensor, DNA from *Salmonella* cultures was mixed with that of related bacteria (*Escherichia coli*) and tested with the NTS *Salmonella* biosensor. Figure 5-31 shows the gel electrophoresis image of PCR amplified DNA from an overnight *S.* Enteritidis mixed 1:1 with generic *E. coli* culture, as well as non-specific bacterial cultures (only *E. coli*). In the lane EC, no DNA bands were observed. Hence, there was no PCR amplification of the *E. coli* genome, indicative of the specificity of the PCR primers used. The thickness of the PCR-amplified '*Salmonella-E. coli* mix' DNA bands increased with the amount of culture inoculum used for PCR (1-5 µl). The DNA bands in lanes S1-S5 were observed parallel to the 500 bp band in marker lane (M).

For specificity testing, extracted genomic *E. coli* DNA as well as the mixture of *Salmonella* and *E. coli* DNA was allowed to hybridize with the NTS biosensor. The signal generated was compared statistically against negative and positive control samples of *Salmonella* Enteritidis DNA to determine the specificity of the biosensor.

5.5.2 Cyclic Voltammograms

Figure 5-32 shows the CV curves for the NTS biosensor chips tested with genomic *E. coli* DNA at 1 ng/µl and 1 pg/µl concentrations obtained by plotting mean current values against the corresponding applied potential. For comparison, the CVs for 1 ng/µl

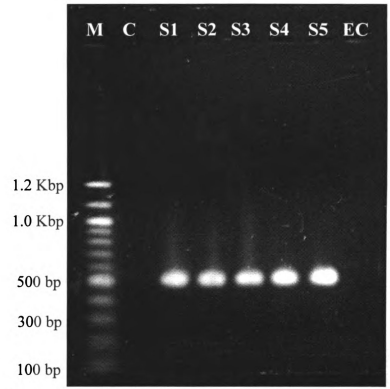

Figure 5-31. Gel electrophoresis of PCR-amplified *Salmonella* and extracted *E. coli* DNA. S1 to S5 – 1 to 5 µl *Salmonella-E. coli* (1:1) culture mix used for PCR. Key: M – Marker, C – negative control, S – *Salmonella-E. coli*, EC – *E. coli*

and 1 pg/µl of *Salmonella* are included along with the CVs for the two *E. coli* DNA concentrations. The anodic current for the CV before DNA probe immobilization (Line 1) was the highest of all the samples tested with the NTS biosensor platform. The CV for the NTS biosensor chip after DNA probe immobilization (Line 6) was significantly lower (as with all previous experiments) than the CV prior to probe immobilization. After the *E. coli* non-target DNA hybridization at both levels were tested (Lines 4 and 5), anodic current did not increase, as it did with *Salmonella* DNA previously (Section 5.4.1.2 above and Figure 5-32), because there was no hybridization of a complementary target to the NTS biosensor chip. However, when a 1:1 mixture of 1ng/µl solution of *Salmonella* and *E. coli* DNA was tested, an increase in the magnitude of the anodic current from that of

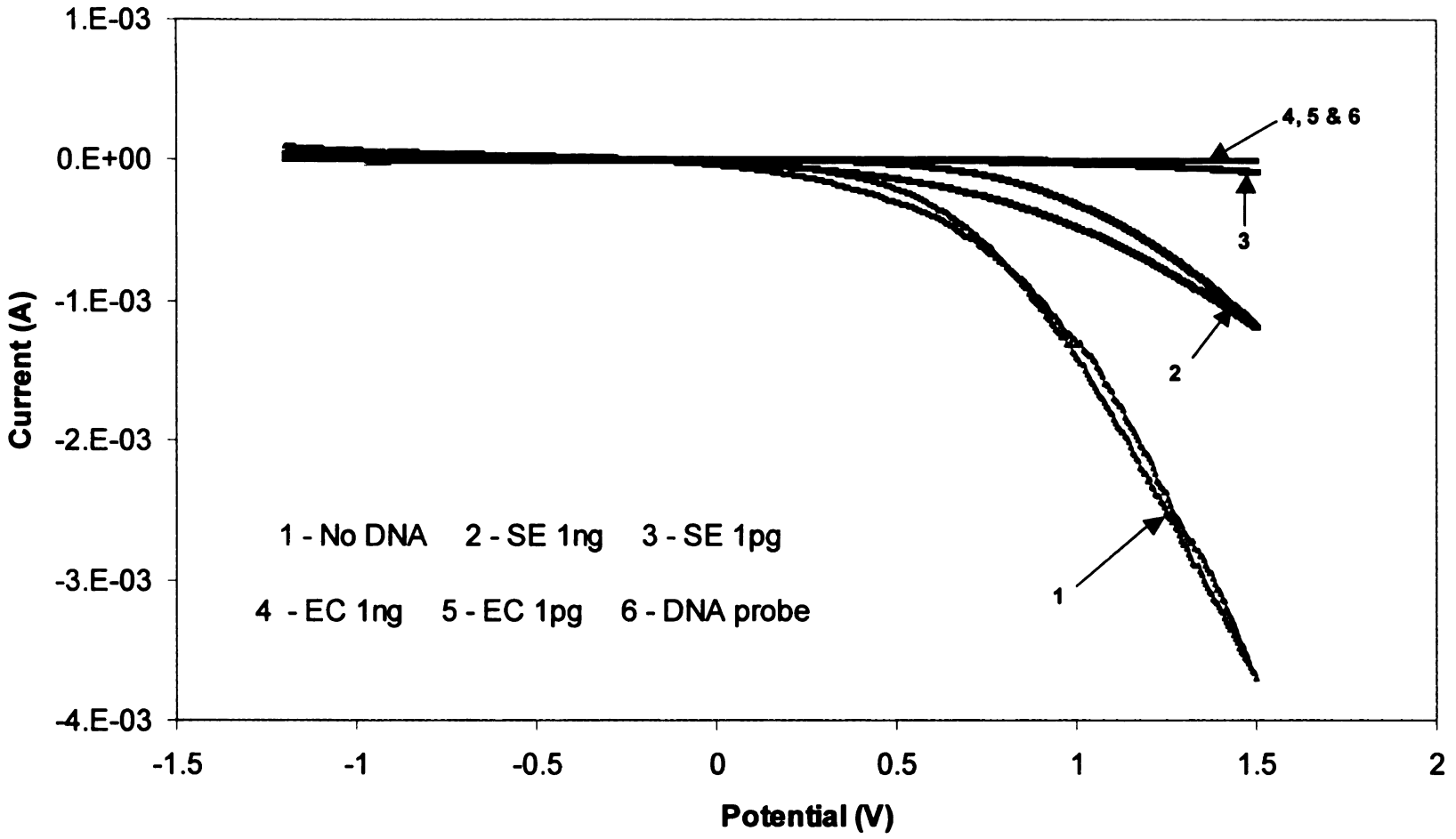

Figure 5-32. Comparison of cyclic voltammograms for *Salmonella* Enteritidis (target) DNA and *E. coli* (non-target) DNA obtained with NTS-based DNA biosensor chip (specificity testing).

the DNA probe chip was observed (Figure 5-33). Thus, even in the presence of non-target DNA, the NTS biosensor was able to hybridize with complementary DNA strands and detect the hybridization event successfully.

5.5.2.1 Analysis of Delta Q values

Delta Q values for CVs of pure culture *Escherichia coli* DNA as well as the *E. coli-S.* Enteritidis mixture (cycle 1 and cycle 2, respectively) at DNA concentrations tested using the NTS biosensor chip are presented in Table 5-11 and Table 5-12. The values of ΔQ for cycles 1 and 2 were analyzed separately as before. Results of ANOVA performed on the ΔQ values are shown in the tables. When the genomic *E. coli* DNA was tested (both 1 ng/μl and 1 pg/μl), there was no increase in the ΔQ values for CVs corresponding to those 'After' DNA target hybridization as compared to that 'Before' target hybridization. For the *Salmonella-E. coli* DNA, in both cycles, the ΔQ values for CVs corresponding to those 'After' DNA target hybridization increased in magnitude compared to that 'Before' target hybridization. For example, for cycle 1, the mean ΔQ values for CVs were -0.456±1.25 mC 'Before' hybridization, and -43.467±1.25 mC 'After', respectively ($p<0.05$). This similar trend was observed in Cycle 2 as well. This change in ΔQ values for *Salmonella-E. coli* DNA in Cycle 1 and Cycle 2 was similar to that observed earlier with PCR-amplified (Section 5.4.1) and purified (Section 5.4.2) *Salmonella* DNA.

The NTS biosensor chips could detect extracted pure culture *S.* Enteritidis DNA even in the presence of non-target *E. coli* DNA at an equally high concentration in the sample. The biosensor was also highly specific to the target, both with the PCR amplification as well as biosensor detection.

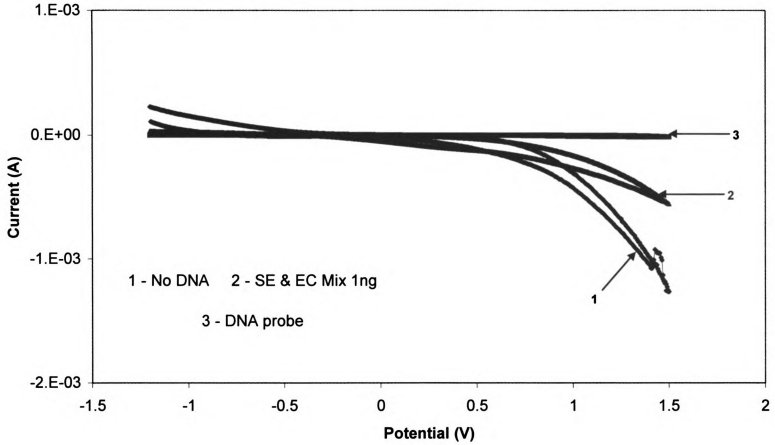

Figure 5-33. Cyclic voltammogram for a mixture of *Salmonella* Enteritidis and *E. coli* DNA obtained with NTS-based DNA biosensor chip (specificity testing).

Table 5-11. Integral charge (ΔQ, milli coulombs) values for *E. coli*, and *S.* Enteritidis-*E. coli* mixture (extracted) DNA with NTS biosensor using cycle 1 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
E. coli 1 ng	-102.667±13.92[b]	-0.261±13.92[a]	-0.083±13.92[a]
E. coli 1 pg	-76.433±13.94[b]	-0.048±13.94[a]	-0.046±13.94[a]
EC/SE mix 1 ng	-52.800±1.25[c]	-0.456±1.25[a]	-43.467±1.25[b]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Table 5-12. Integral charge (ΔQ, milli coulombs) values for *E. coli*, and *S.* Enteritidis-*E. coli* mixture (extracted) DNA with NTS biosensor using cycle 2 of cyclic voltammogram.

	DNA Hybridization Condition		
Concentration	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
E. coli 1 ng	-93.567±14.36[b]	-0.240±14.36[a]	-0.076±14.36[a]
E. coli 1 pg	-49.833±8.00[b]	-0.038±8.00[a]	-0.048±8.00[a]
EC/SE mix 1 ng	-35.600±1.74[c]	-0.181±1.74[a]	-23.733±1.74[b]

Different superscripts for means across the row indicate significantly different charge values ($p<0.05$).

Thus, the NTS biosensor could potentially be used with environmental samples that normally have a high load of background DNA, protein, and other organic matter that could interfere with the biosensor performance. However, this would require fabrication of the NTS sensor platform with highly consistent pore characteristics (diameter and thickness). Current results of the DNA-based NTS biosensor show high degree of variability in performance. These differences could be caused by variability in chip

characteristics, source of DNA, and sample matrix being tested. For example, ΔQ values obtained with 1 ng of PCR-amplified and purified (extracted) *Salmonella* DNA (Objective 4) as well as that for 1 ng of *Salmonella-E. coli* DNA (Objective 5) were signinficantly different from each other (Table 5-13 and Table 5-14). The high variability in output data is a barrier that will need to be overcome for successful development of a commercial DNA-based NTS biosensor.

Table 5-13. Integral charge (ΔQ, milli coulombs) values for *S.* Enteritidis DNA from different sources with NTS biosensor (Cycle 1).

	DNA Hybridization Condition		
DNA Source	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
PCR 1 ng	-173.333±3.72^{e}	-0.231±3.72^{a}	-45.767±3.72^{c}
Extracted 1 ng	-30.633±0.81^{b}	-0.028±0.81^{a}	-75.700±0.81^{d}
EC/SE mix 1 ng	-52.800±1.25^{cd}	-0.456±1.25^{a}	-43.467±1.25^{c}

Different superscripts for means indicate significantly different charge values ($p<0.05$).

Table 5-14. Integral charge (ΔQ, milli coulombs) values for *S.* Enteritidis DNA from different sources with NTS biosensor (Cycle 2).

	DNA Hybridization Condition		
DNA Source	Blank, ΔQ (mC)	Before, ΔQ (mC)	After, ΔQ (mC)
PCR 1 ng	-148.000±2.70^{c}	-0.202±2.70^{a}	-39.667±2.70^{b}
Extracted 1 ng	-45.133±0.99^{c}	-0.030±0.99^{a}	-28.333±0.99^{b}
EC/SE mix 1 ng	-35.600±1.74^{c}	-0.181±1.74^{a}	-23.733±1.74^{b}

Different superscripts for means indicate significantly different charge values ($p<0.05$).

CHAPTER 6: CONCLUSION

A versatile silicon-based biosensor platform for pathogen detection was fabricated. Silicon (0.01ohm-cm, p-type) was etched in an electrochemical cell containing hydrofluoric acid solution using anodizing conditions of 5 mA/cm^2 for 1 h to fabricate NTS. NTS chips were functionalized into a biosensor using biological sensing elements such as enzymes, antibodies, and DNA probes. A chemiluminescence-based enzyme assay that could detect 10^3 CFU was adapted to the biosensor system for detection of *E. coli* with a lower detection limit (sensitivity) of 10^2 CFU within 60 min. The NTS-based platform was also functionalized into an immuno-biosensor to successfully detect a pure culture of *Salmonella* Typhimurium within 10 min. Finally, a DNA-based NTS biosensor was developed using an electrochemical transducer for detection of *Salmonella* Enteritidis. The NTS DNA biosensor was able to detect *S.* Enteritidis DNA with a lower limit of detection of 1 pg/μl for PCR-amplified DNA and 10 pg/μl for pure culture-extracted DNA. The electrochemical detection process was completed within 60 min. In comparison, the planar Si DNA biosensor could not detect *S.* Enteritidis at 1 ng/μl level or lower tested. Hence, the NTS biosensor had better performance in terms of lower limit of detection for the target with both, the enzyme assay as well as the DNA-based detection assay. The versatility of the NTS platform for pathogen detection was successfully demonstrated using three different biological sensing elements and two detection methods.

CHAPTER 7: FUTURE RESEARCH

Future research activities should focus on:

1. Fabricating NTS platform with highly consistent pore characteristics.
2. Developing the NTS immunobiosensor for detection of viral particles in food samples, and antibodies or other small biological molecules of importance (for example, food allergens).
3. Investigating the mechanisms responsible for the intrinsic electrochemical properties of DNA that can be exploited in biosensing applications to improve lower limits of DNA-based detection.
4. Developing the NTS DNA biosensor at the nano-level to increase the sensitivity while exploiting the intrinsic electrochemical properties of DNA as was demonstrated in the current research at a macro level.
5. Developing enzyme-based assays for detection of pathogens other than *E. coli* O157:H7 and *E. coli*/coliforms by identifying unique markers for targets of interest.
6. Developing a multi-array biosensor device with multiple biological sensing elements and transduction mechanisms to fabricate a self-validating detection device.
7. Wireless transmission of data for the field ready biosensors for centralized data collection/analysis with potential application in areas like epidemiology or on-site testing.

APPENDICES

APPENDIX A: PROCEDURES

A.1 FRESH PRODUCE TESTING

Samples of lettuce (Romaine, Boston, green leaf, red leaf), sprouts (clover, alfalfa, bean, broccoli), green onions, parsley, spinach, tomatoes (cherry and grape), shredded carrots, cabbage, salads (spring mix, coleslaw, herb, garden), strawberries, raspberries, blackberries, cantaloupes, and apple cider were obtained from various local grocery shops that included three supermarkets, organic food stores and farmer's market. The produce samples were used for the assay without any treatment, such as washing, that would alter the natural microflora. All samples were processed as shown in Figure A-1.

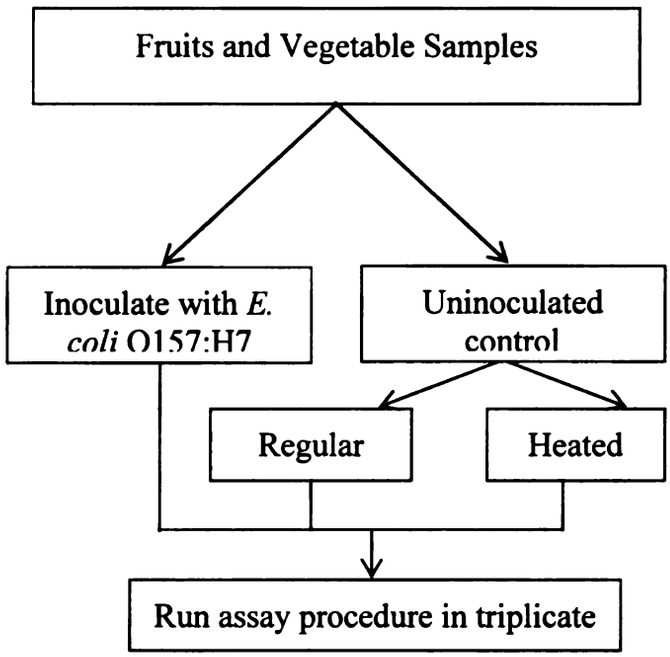

Figure A-1. Experimental design for processing of produce samples

Eleven ml of apple cider was transferred into sterile test tubes, inoculated with 0.1 ml of serially diluted *E. coli* O157:H7 culture and used for the assay. Produce samples were weighed (11 gm each or whole fruit), placed in sterile 4 oz Whirl-Pak bags and followed by addition of serially diluted *E. coli* O157:H7 cultures (to differentiate from

natural *E. coli* contamination) at different contamination levels (10^2-10^6 colony forming units/g [CFU/g] of produce). Uninoculated produce samples from the same package were used as the control (or blank) to determine the natural *E. coli* contamination levels. The samples were stored for 45 min-1 h to permit attachment of the bacterial cells on the produce surface. Based on the sample size, 0.1 % peptone solution was added to obtain a 1:10 dilution. Strawberry samples were rubbed gently on the surface, whereas all other samples were pummeled in Stomacher® 80 (Seward Ltd., London, UK) at 30 rpm, to extract bacteria from the produce surface into the peptone. A tenth of the total sample volume was removed from the Whirl-Pak bag and used for the assay as described by Mathew and Alocilja (2002). For the uninoculated samples, a tenth of the total volume was removed separately into a test tube, heated in a boiling water bath for 10 min, cooled and used for the assay. The heating killed *E. coli* present in the sample and denatured any β-galactosidase enzyme produced by them. The difference in light emission, measured using the Femtomaster luminometer, between the heated and uninoculated control was used to determine the level of natural contamination from β-galactosidase producing organisms. Sensitivity of the assay was determined by comparing the light emission data collected at the third 10-min interval (established as ideal based on prior results) with *E. coli* and *E. coli* O157:H7 counts obtained from SMAC plates for all samples. All trials were done in triplicate.

A.2 S-4700II Scanning Electron Microscope Operation

Startup

Make sure that the vacuum is OK and the vacuum gauge readings are normal - IP1: $1x10^{-8}$, IP2: $1x10^{-7}$, IP3: $1x10^{-6}$, Pe (S.C.): L - $1x10^{-3}$

Loading the sample

Prior to loading the sample, ensure that the HV (high voltage) is OFF, SEM stage lock is released (gray), the stage is at home position (green) by clicking on the GOTO HOME button on Stage Control window, VALVE/GUN (gun isolation valve) is at the CLOSE position, and CHAMBER switch is at the S.E.C. (sample exchange chamber) position. Mount a sample on the sample holder. Use the height gauge to set the proper height of the sample for correct WD reading. Press the AIR button to vent the S.E.C. Once the S.E.C. is opened, mount the sample holder on to the exchange rod. Do not over tighten the sample holder. Close and hold the S.E.C. by hand, then press the EVAC button to pump down the S.E.C. When the green HIGH lamp of the S.E.C. VACUUM is on, and the yellow VALVE/EXCHANGE OPEN light flashes, the sample is ready for transfer into the sample chamber (S.C.). Open the S.E.C. isolation valve (MV1) and transfer the sample onto the SEM stage. The chamber scope should be turned on to monitor the sample mounting process. Withdraw the sample exchange rod and close the S.E.C. isolation valve.

Viewing the sample

When the yellow VALVE/GUN AUTO light is flashing, set the VALVE/GUN switch to AUTO (OPEN), which links the operation of the isolation valve to the high voltage. Select the desired accelerating voltage (15KV) and emission current (10 uA) in

the high voltage control window. Press the ON button to turn on the high voltage. If program requests a tip flashing (regenerating), see the next section. The SEM image should be viewable.

Tip flashing

Residue molecules in the UHV gun chamber will cover the surface of the field emission tip over time, reducing the emission current and the probe current stability. To revitalize the emission tip, a flashing procedure needs to be carried out on a regular basis. To flash the tip, the high voltage has to be OFF. Press the FLASHING button on the HV Control window. After flashing, the emission current undergoes a drift period for about 30-60 min and then remains stable for a number of hours.

Beam adjustment

For best resolution, alignment of the beam, the objective aperture, and the stigmators, is necessary.

Sample removal from the SEM

Turn off the high voltage. Put the VALVE/GUN switch back to CLOSE position. With Stage Control window active, click GO TO HOME button with stage lock released. Open the S.E.C. isolation valve (MV1), and use the sample exchange rod to remove the sample. Do NOT over tighten the thread on the sample exchange rod. Pull the rod all the way out and close the MV1 valve. Make sure the S.E.C. switch is at S.E.C. position. Vent the S.E.C. by pressing the AIR button, and remove the sample.

A.3 GEL ELECTROPHORESIS

Casting agarose gels using Embi Tec RunOne System gel trays

The gel casting stands and trays were cleaned before each use. The casting stand was placed on a level surface to ensure uniform gel thickness. The agarose gel trays were then

placed in the casting stand. A 1.3 % agarose solution was prepared by adding 1.3 g of agarose (Sigma Chemicals) to 100 ml of deionized water, boiling the solution to completely dissolve the agarose, followed by cooling to 50°C. For each gel, 15 ml of the 1.3 % agarose solution was carefully poured into the gel tray yielding a gel 4.5 mm thick. Any air bubbles in the gel solution were carefully removed using a sterile pipette tip. The 10 µl-volume comb was inserted into the gel solution, and the gel allowed to solidify for at least 30 minutes.

Running the gel using the RunOne Gel System

The agarose gel tray with cover was placed on the running platform of the RunOne Cell. The gel was oriented so that the wells were next to the Power Supply on the right side of the tank. The wells were flushed with 1X Tris-Borate EDTA (TBE) running buffer to remove any debris. 300 ml of the 1X TBE running buffer was poured into the RunOne tank to ensure that the agarose gel tray cover was fully submerged. The DNA samples were prepared for loading into the wells by premixing 5 µl of the DNA with 5 µl of 1X loading dye (see Appendix B). The samples were loaded into the wells with the 100 bp DNA marker (New England Biolabs, Inc., Beverly, MA) in the first lane and the DNA samples in the rest of the lanes. The tank lid was placed on the RunOne Cell. The 'VOLTAGE SELECT' button was pressed to choose the desired voltage output (75 V) and power turned ON to begin electrophoresis. DNA, being negatively charged, migrates from the cathode (-) to anode (+). The power supply was turned OFF when electrophoresis was finished (45 min).

Staining Gels with Ethidium Bromide

WARNING: Ethidium bromide (EtBr) is a known mutagen and suspected carcinogen. Always wear gloves and safety glasses when handling it. Follow appropriate hazardous materials disposal regulations.

Ten microliters of a 10 mg/ml stock solution of EtBr solution was added to 100 ml of TBE running buffer water in the staining container. The EtBr solution was allowed to mix for at least one minute for uniform dispersion. Each gel was placed in the staining container to stain for 15 minutes. After staining, the gel was removed carefully and placed in a destaining container (100 ml of TBE running buffer) to destain the gel for 10 minutes. The DNA was visualized using a short wave (254-314 nm) UV transilluminator in a UVT tray. Images of the gel were obtained using a Kodak Gel Imaging system.

APPENDIX B: REAGENTS/SOLUTIONS

B.1 Reagents for Enzyme-Based and Antibody-Based Testing

Dilution blanks:

Add 1.0 g of Peptone Digest (Bacto Peptone from Difco) to 1 L of distilled/deionized water and dispense slightly greater than 9.0 ml (9.05 or 9.1) into test tubes (that takes care of losses during autoclaving). Autoclave the test tubes at 121°C for 15 min.

Phosphate Buffer (0.02M, pH 7.4):

(Note: Tris-HCl buffer is usually used as carrier buffer for alkaline phosphatase systems)

Find out the molecular weight of Potassium phosphate (dibasic and monobasic)

Calculate grams of the di and mono basic potassium phosphate as follows:

Required Molarity * Mol. Wt. * volume needed, e.g. 0.02*136.1*1.6L = 4.352g in 1.6L

Guideline: Mix 4 volumes of the 0.02M K_2HPO_4 (dibasic) to 1 volume of KH_2PO_4 (monobasic). The best way to do it would be to take the above K_2HPO_4 solution, measure its pH and then add the KH_2PO_4 solution in 25-50 ml increments and measure pH until the final pH is adjusted to 7.4

Blocking solution

Recommendation – 1-3% BSA

Weigh out 1 g of Albumin powder (Sigma Chemicals). Add to 50 ml of phosphate buffer pH 7.4. This will yield a 2% blocking solution.

Wash solution

0.05-0.1% Tween-20 Solution: Weigh out 8.7 g of NaCl. Add to 900 ml of distilled water and dissolve. Add 20 ml of phosphate buffer. Mix well. Add 0.5 ml of Tween-20 solution. Make up volume to 1 L by adding about 80 ml of distilled water. Mix very well so that the Tween-20 is uniformly mixed into this wash solution.

Note: Literature is available where 0.1% Tween-20 solution was used to decrease background noise (so add 1 ml of Tween-20 instead of 0.5 ml in the above procedure).

B.2 REAGENTS FOR DNA-BASED TESTING

Hybridization Buffer

Place 100 ml of the hybridization buffer supplied with the detection system in a beaker containing a magnetic stir bar. Place the beaker on a heating stir plate and rapidly stir the buffer while heating to 42 deg. C. Quickly, add 4.38 g of NaCl and 5.0 g of blocking powder also (provided with the detection system) into the buffer. Continue to stir this mixture at 42 deg. C for 1 h. The buffer may now be aliquoted into sterile plastic tubes and stored at -20 deg. C. Prior to use, the buffer must be thawed and re-heated to 42 deg. C for at least 30 minutes.

20X SSC

Add 175.3 g NaCl and 88.2 g NaCitrate (trisodium salt) in distilled-deionized water to a final volume of 1.0 liter. Adjust the pH of the solution to 7.0 using HCl or NaOH. This solution can be stored at room temperature without sterilization.

20% SDS (sodium lauryl [dodecyl] sulfate)

Dissolve 100 g SDS in 400 ml of distilled-deionized water. The solution will be cloudy, but adjust the pH to 7.2 using HCl. Bring the final volume to 500 ml with distilled-deionized water. This solution can be stored at room temperature without

sterilization. (If the solution remains cloudy or becomes cloudy in the future, warm it to 50-65 deg. C to dissolve the SDS before dispensing.)

Primary Wash Buffer

To 800 ml of distilled-deionized water, add 25 ml of 20X SSC, 20 ml of 20% SDS, and 360 g urea. Bring the final volume to 1000 ml with distilled-deionized water. This solution can be stored at room temperature without sterilization. Note: Stringency can be increased by using only 5 ml of 20X SSC instead of 25 ml. However, in our experience, this has not be found necessary with the IS6110 probe.

Secondary Wash Buffer

To 800 ml of distilled-deionized water, add 100 ml of 20X SSC. Bring the final volume to 1000 ml with distilled-deionized water. This solution can be stored at room temperature without sterilization.

Agarose Gel Loading Dye (6x Loading Dye)

For 250ml:

Add 0.63 g Bromophenol Blue, anhydrous to about 150 ml of water in a 500ml glass beaker, stir for 60min. Add 25 ml of 100% glycerol with a 60cc syringe, stir until thoroughly mixed, an additional 30min. Make up the volume to 250 ml with deionized water. Continue to stir for 30 min. Adjust to final volume of 250 ml. Aliquot 20 ml of dye into 50 ml conical tubes. Store at 4°C.

APPENDIX C: RAW DATA AND STATISTICAL ANALYSIS

C.1 RAW DATA: CYCLIC VOLTAMMETRY

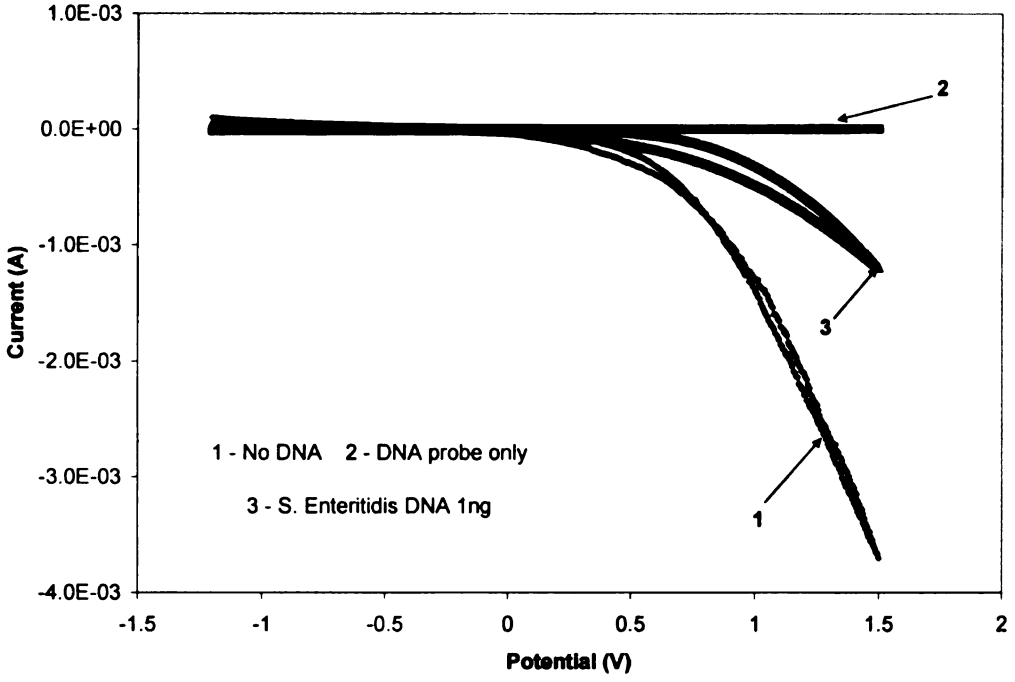

Figure C-1. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 ng of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

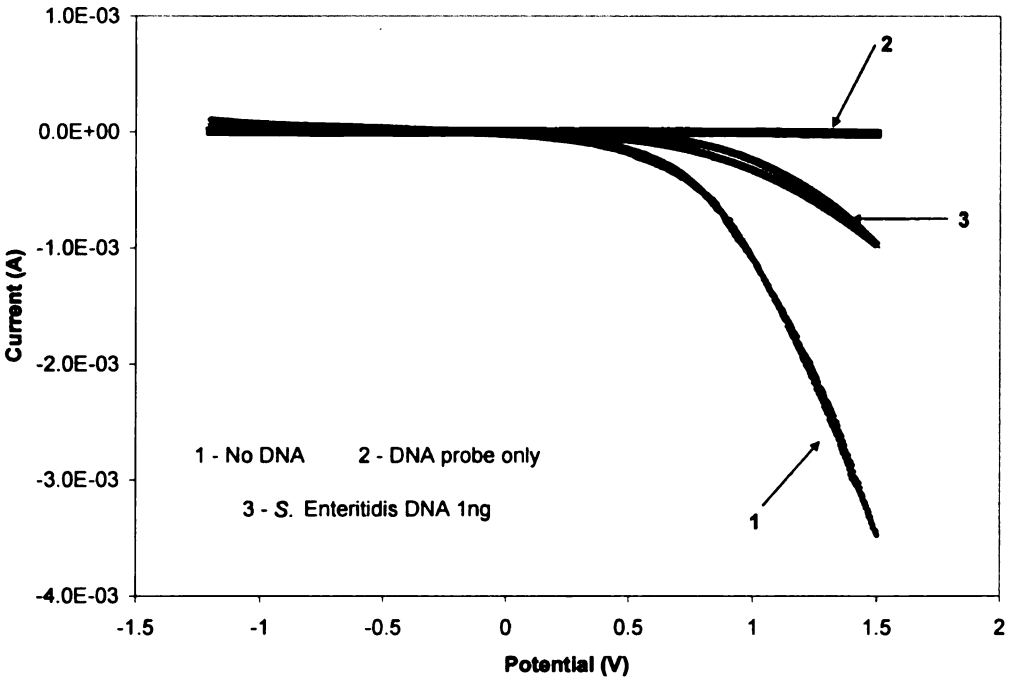

Figure C-2. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 ng of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

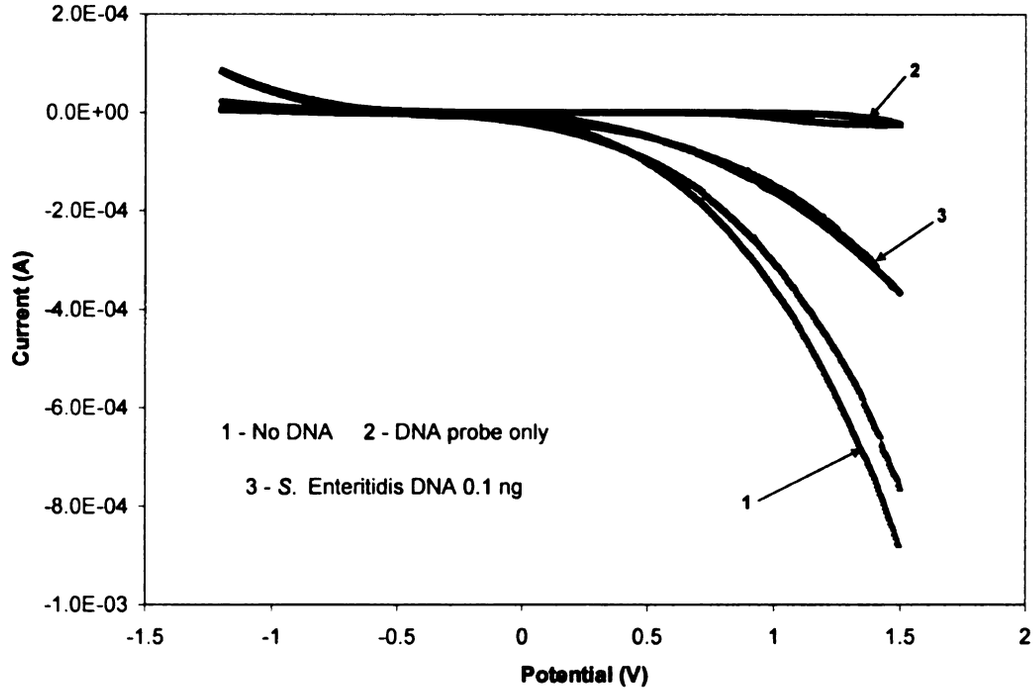

Figure C-3. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 0.1 ng of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

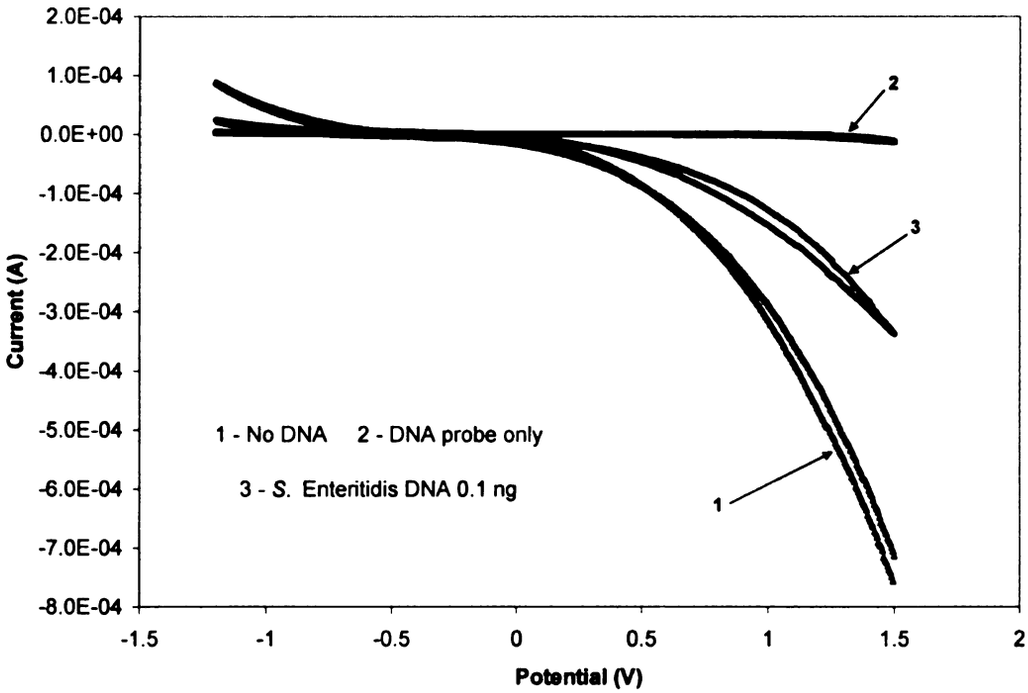

Figure C-4. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 0.1 ng of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

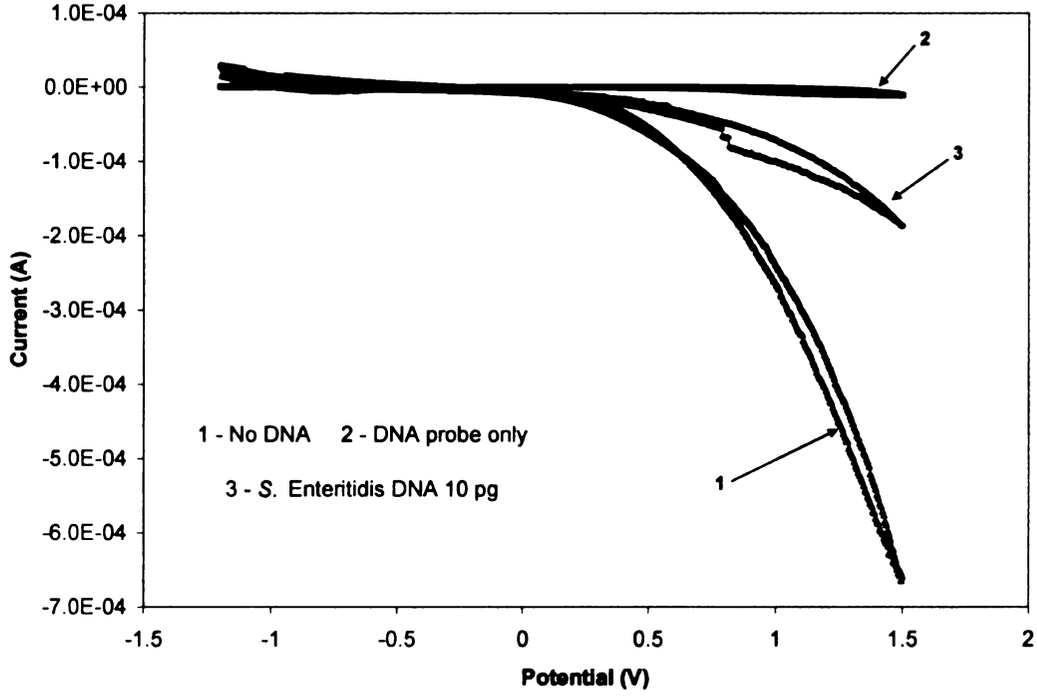

Figure C-5. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 10 pg of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

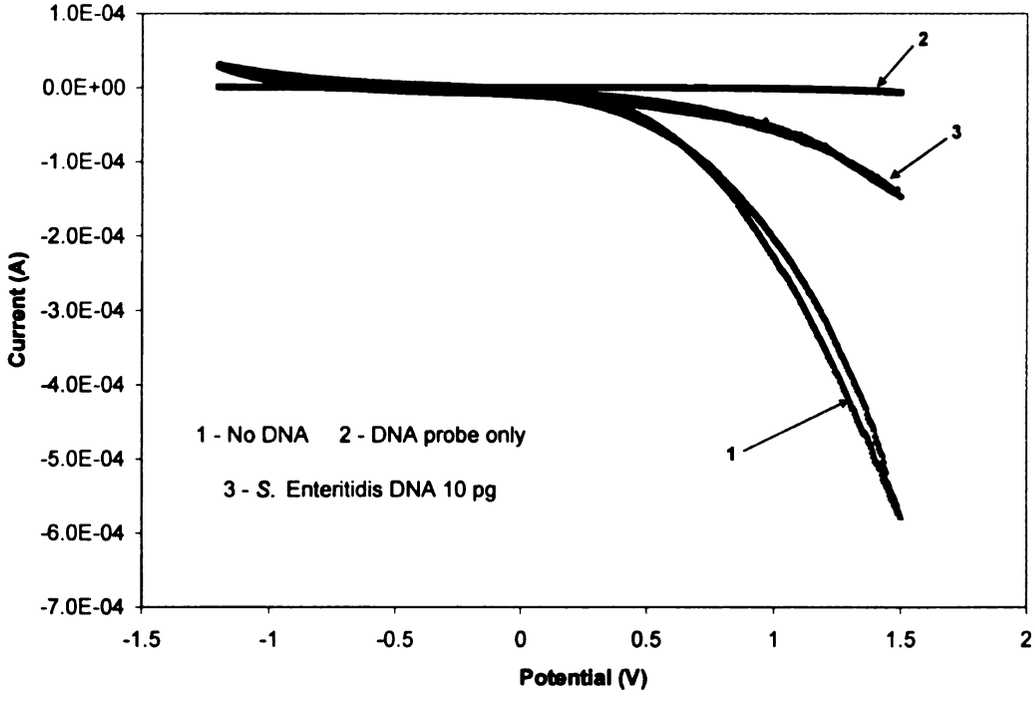

Figure C-6. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 10 pg of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

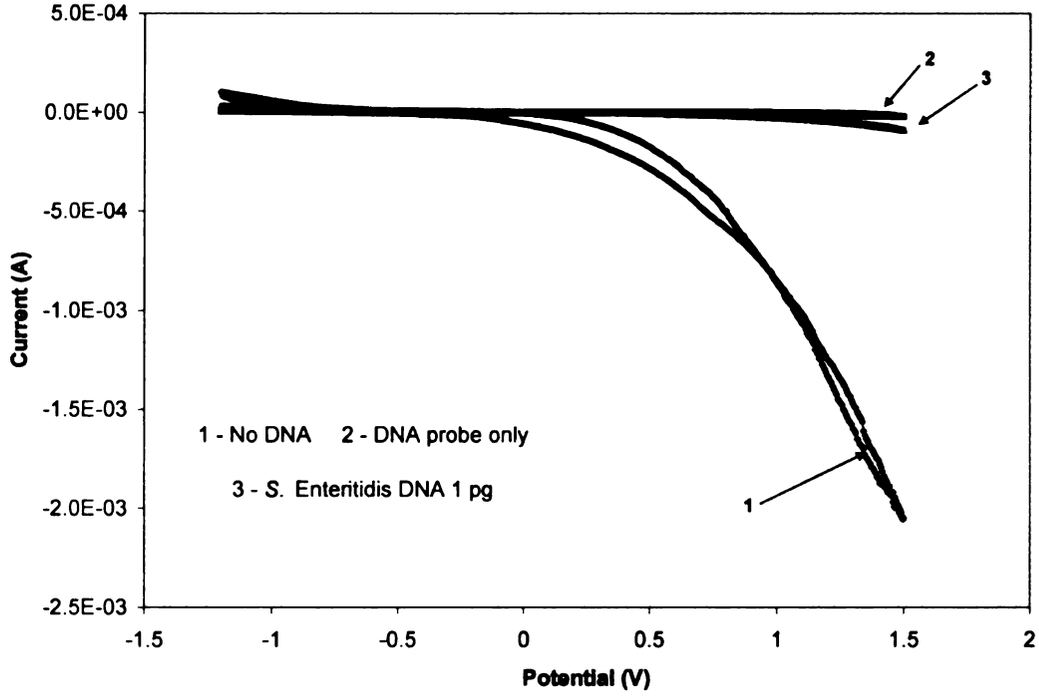

Figure C-7. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 pg of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

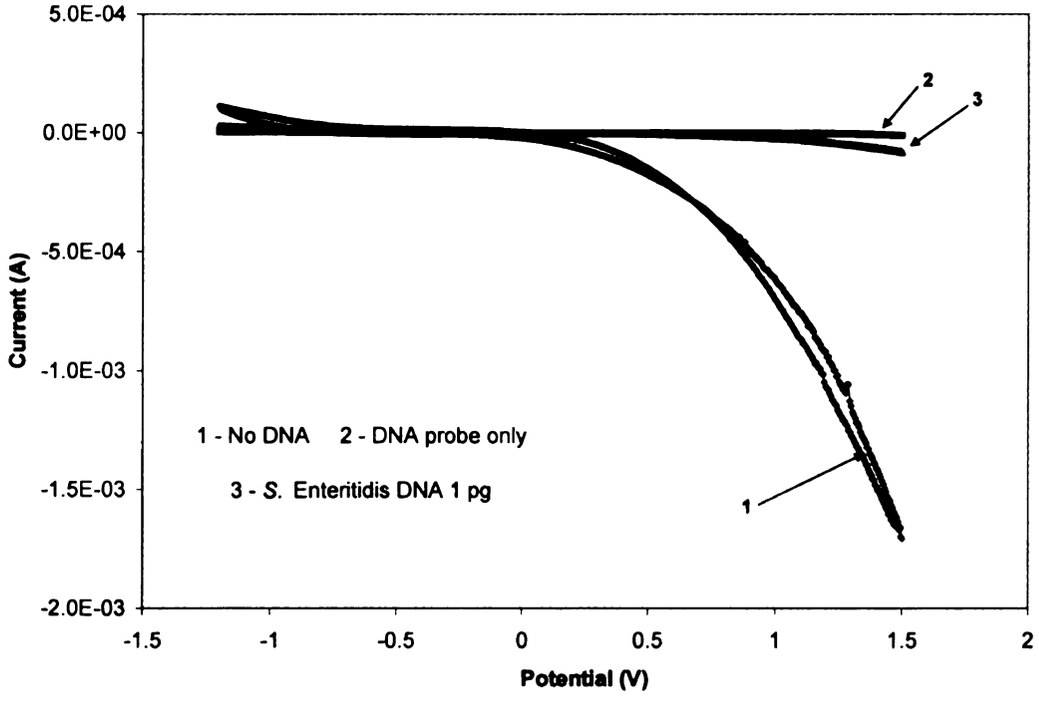

Figure C-8. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 10 pg of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

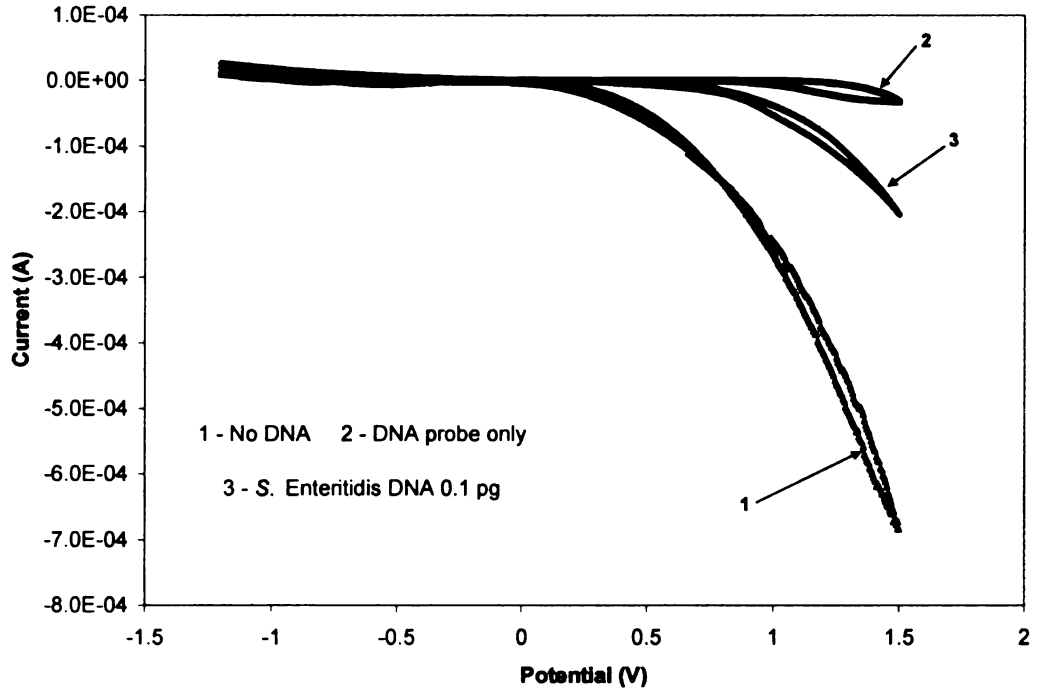

Figure C-9. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 0.1 pg of PCR-amplified *Salmonella Enteritidis* DNA (n = 3).

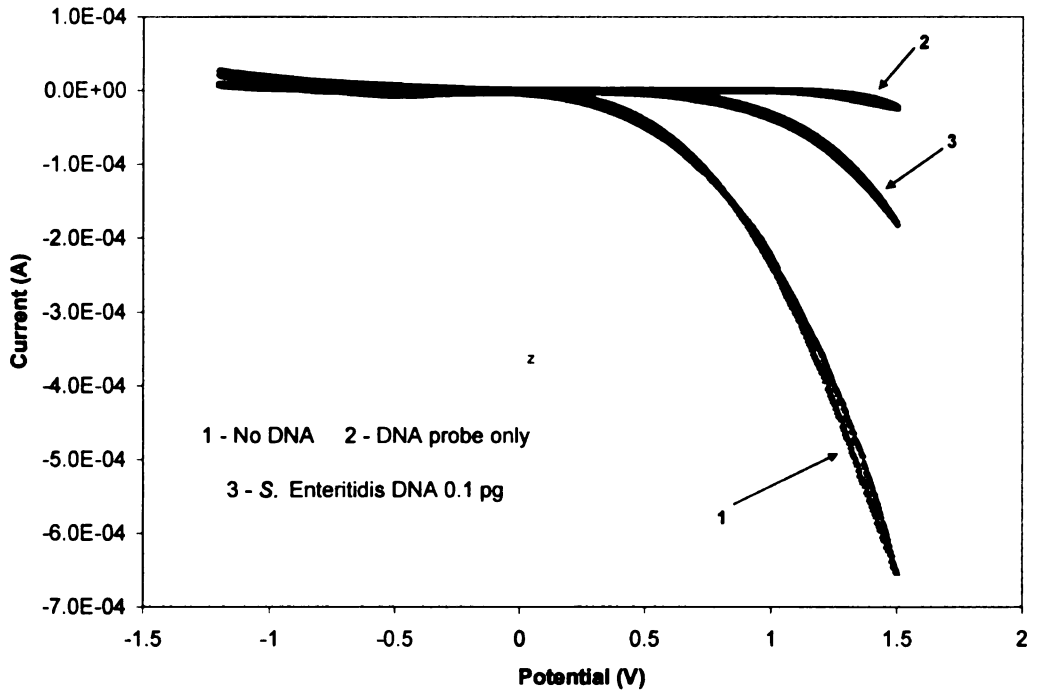

Figure C-10. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 0.1 pg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

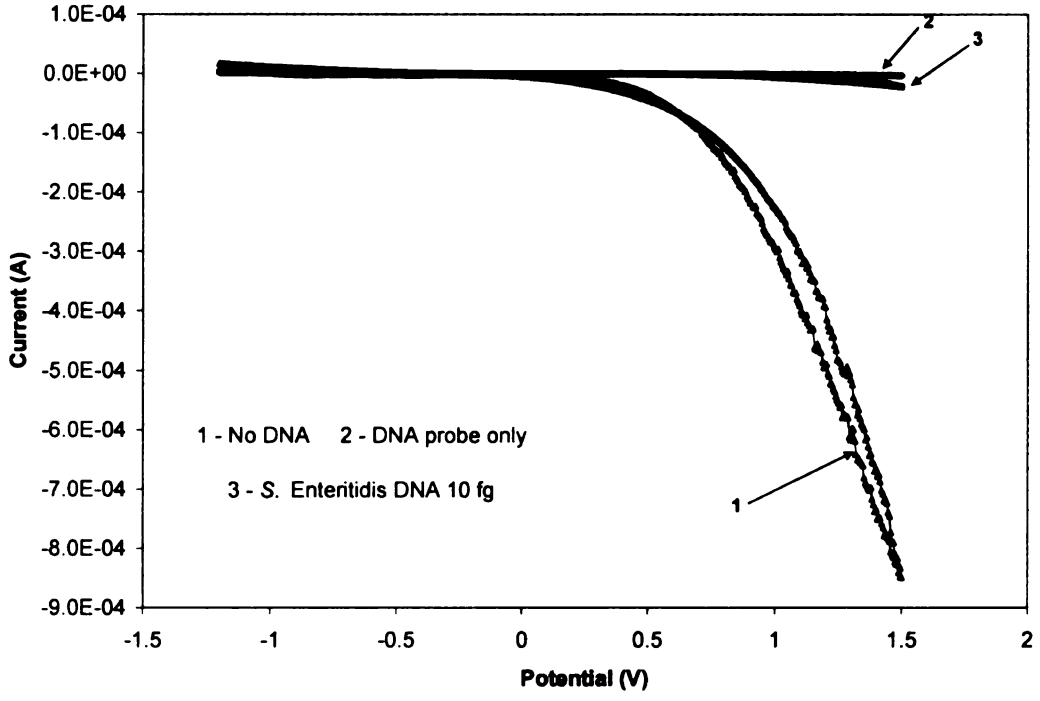

Figure C-11. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 10 fg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

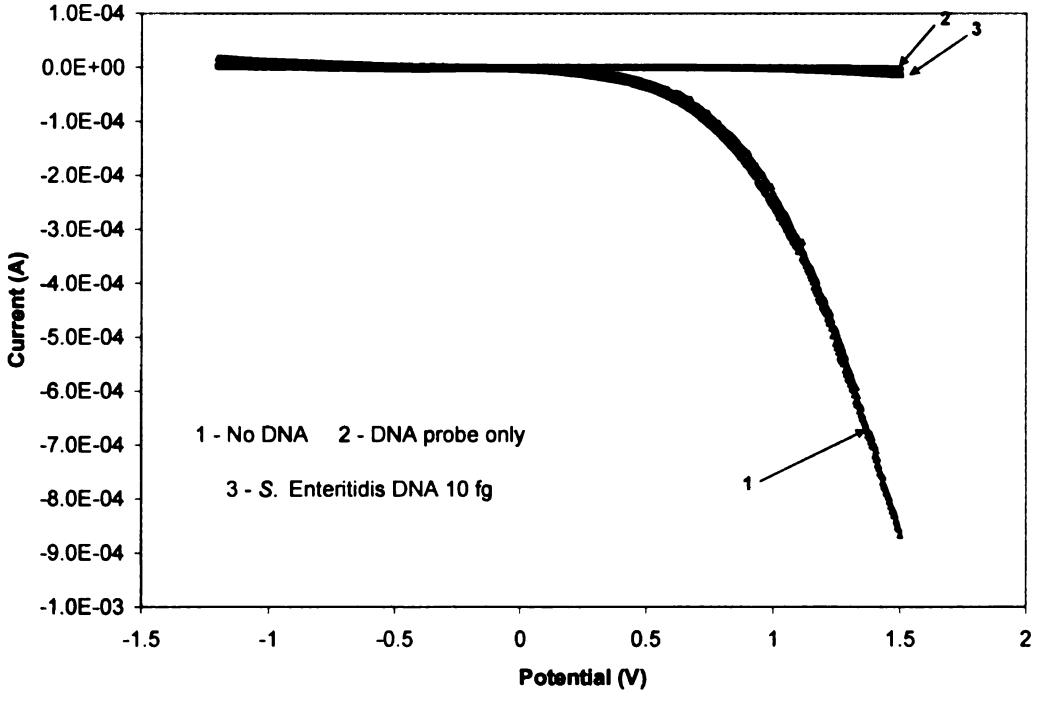

Figure C-12. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 10 fg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

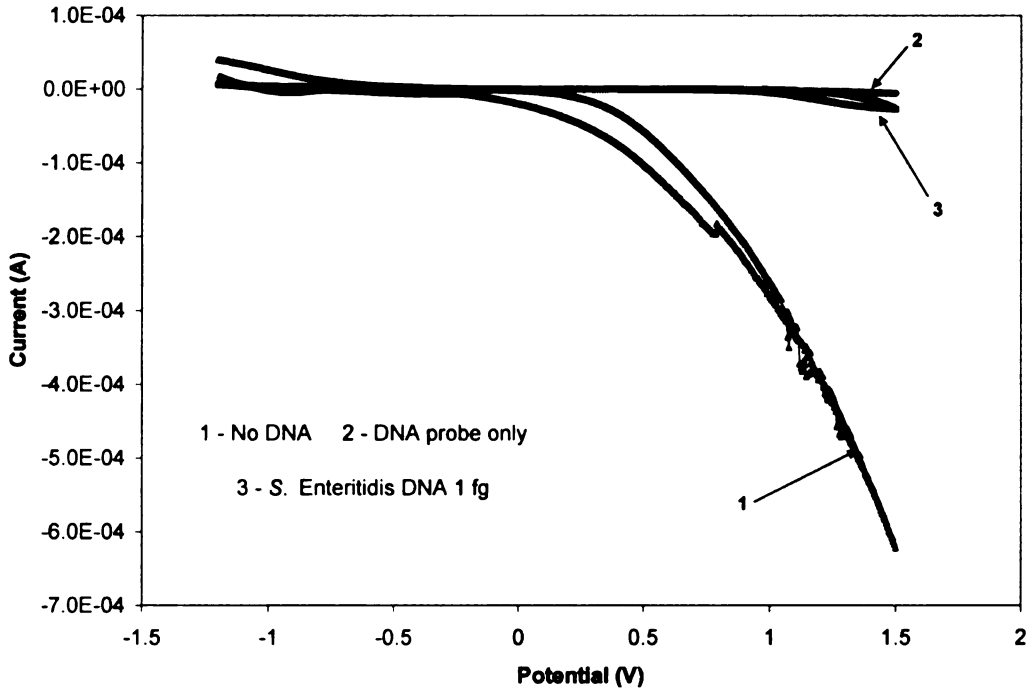

Figure C-13. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 fg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

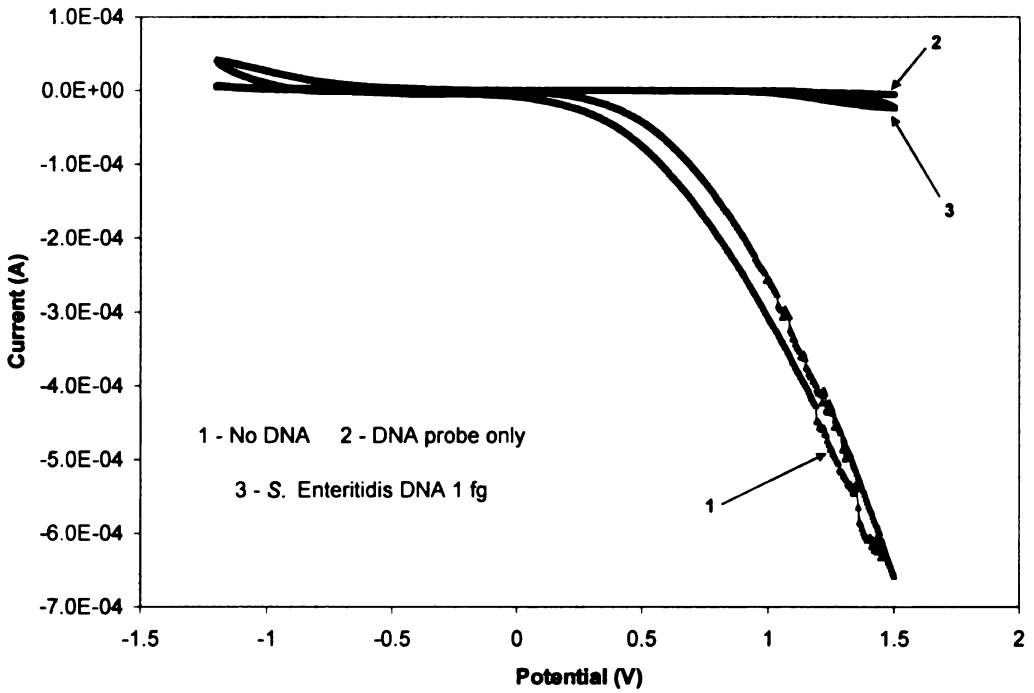

Figure C-14. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 fg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

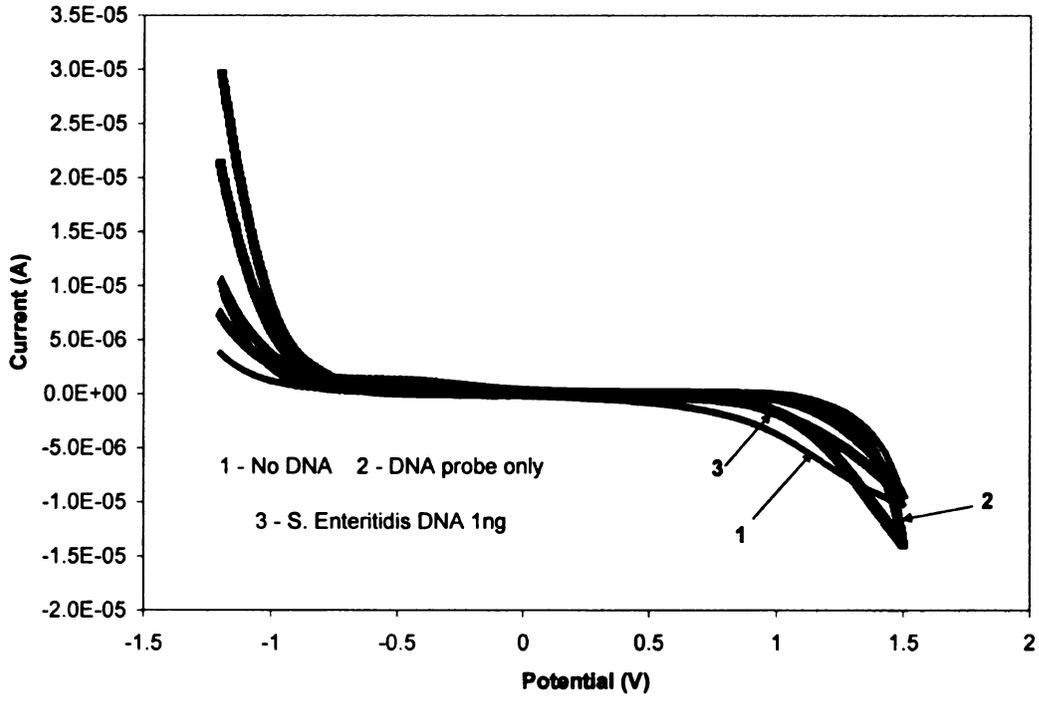

Figure C-15. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor chip for 1 ng of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

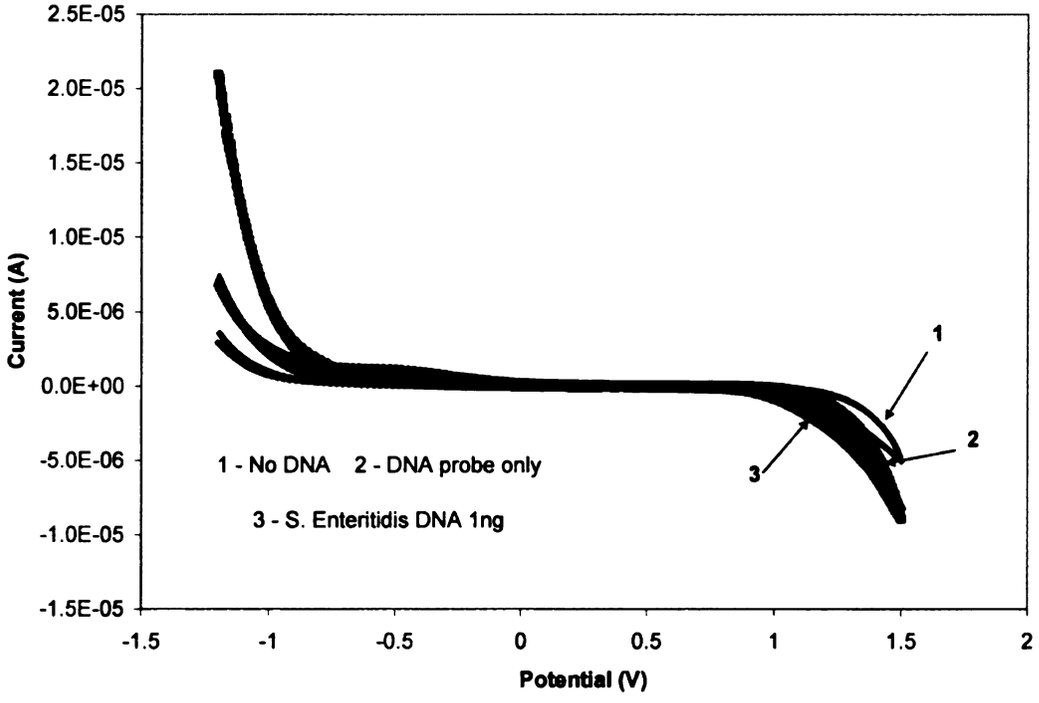

Figure C-16. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor chip for 1 ng of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

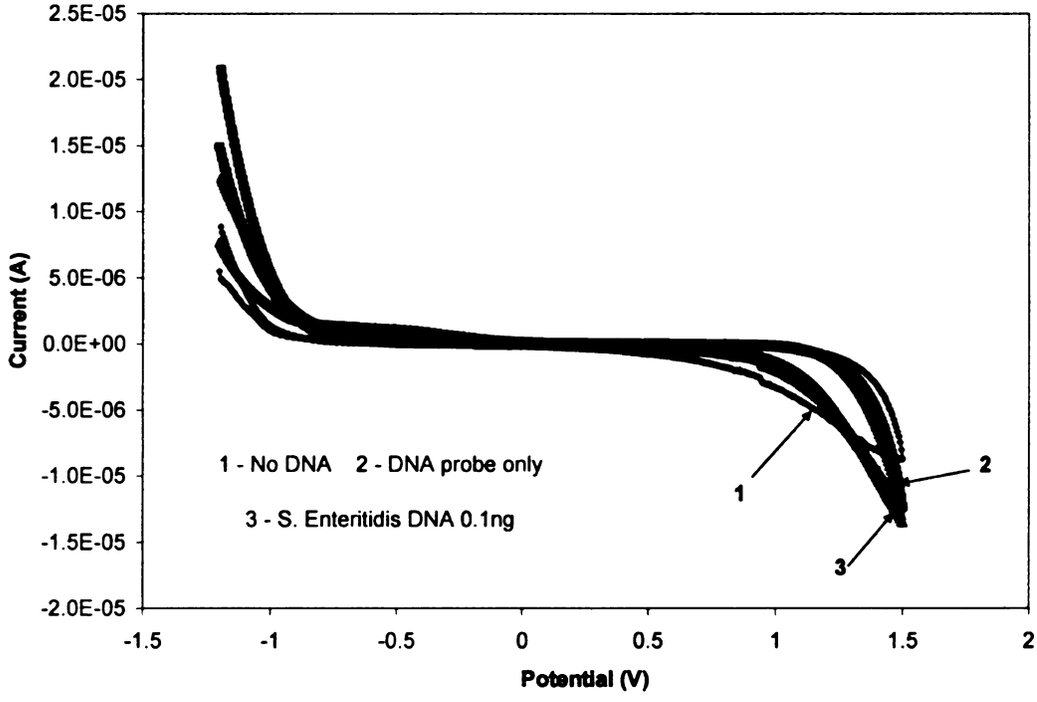

Figure C-17. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor chip for 0.1 ng of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

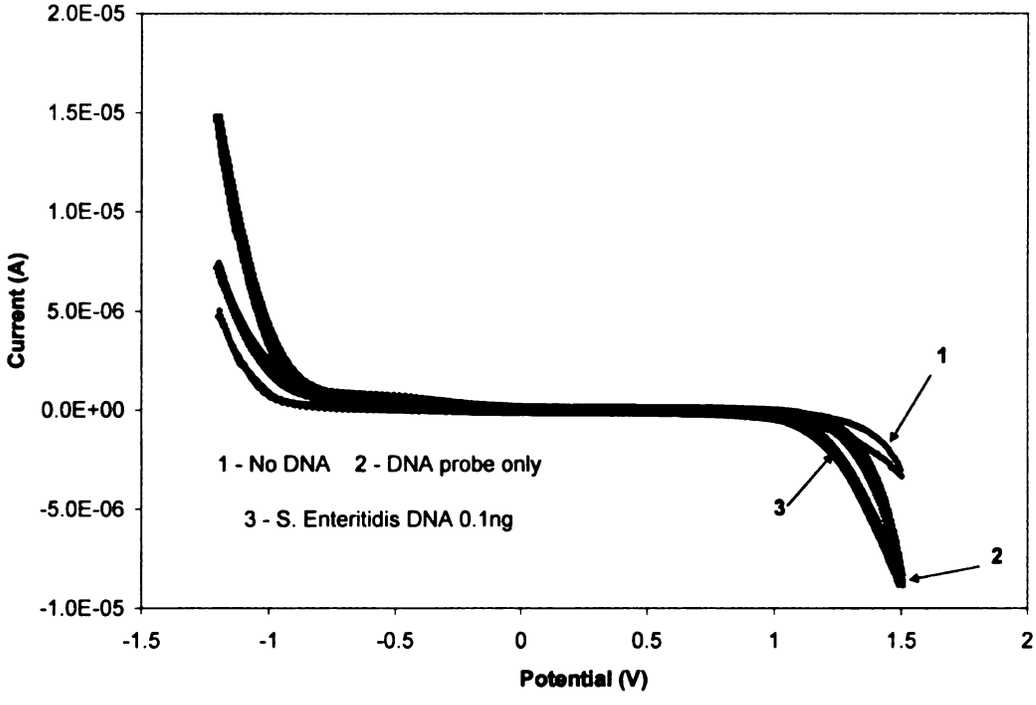

Figure C-18. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor chip for 0.1 ng of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

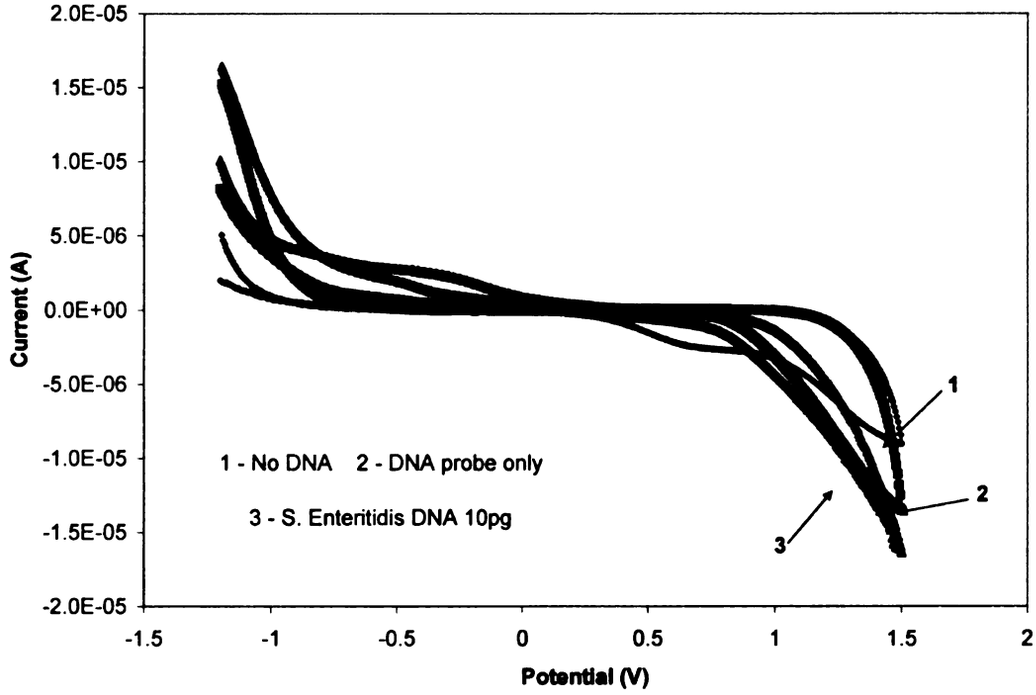

Figure C-19. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor chip for 10 pg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

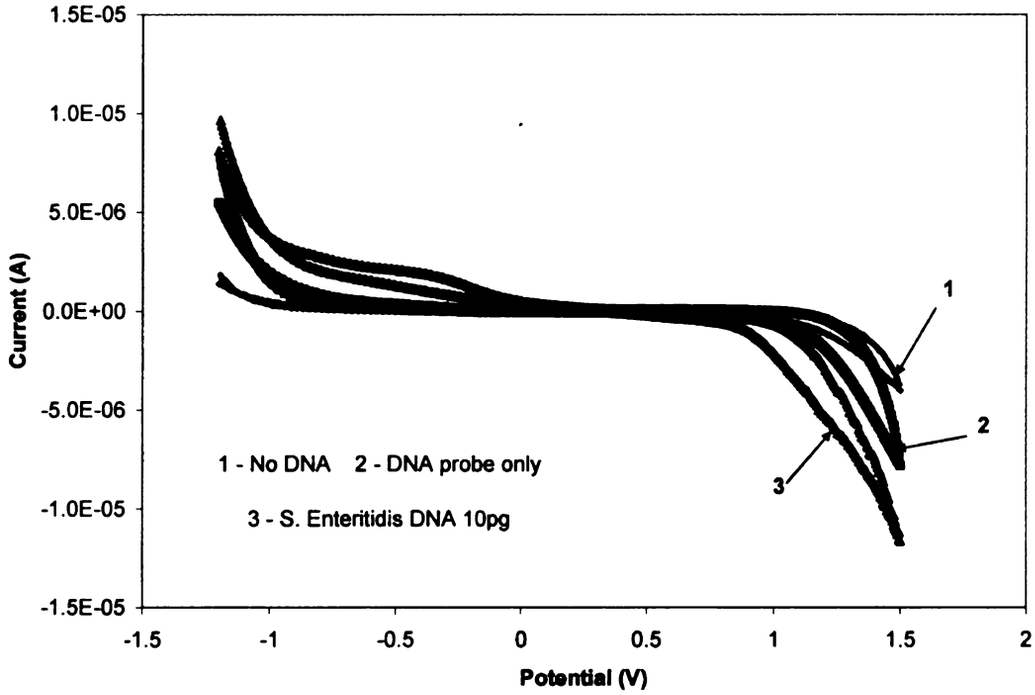

Figure C-20. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor chip for 10 pg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

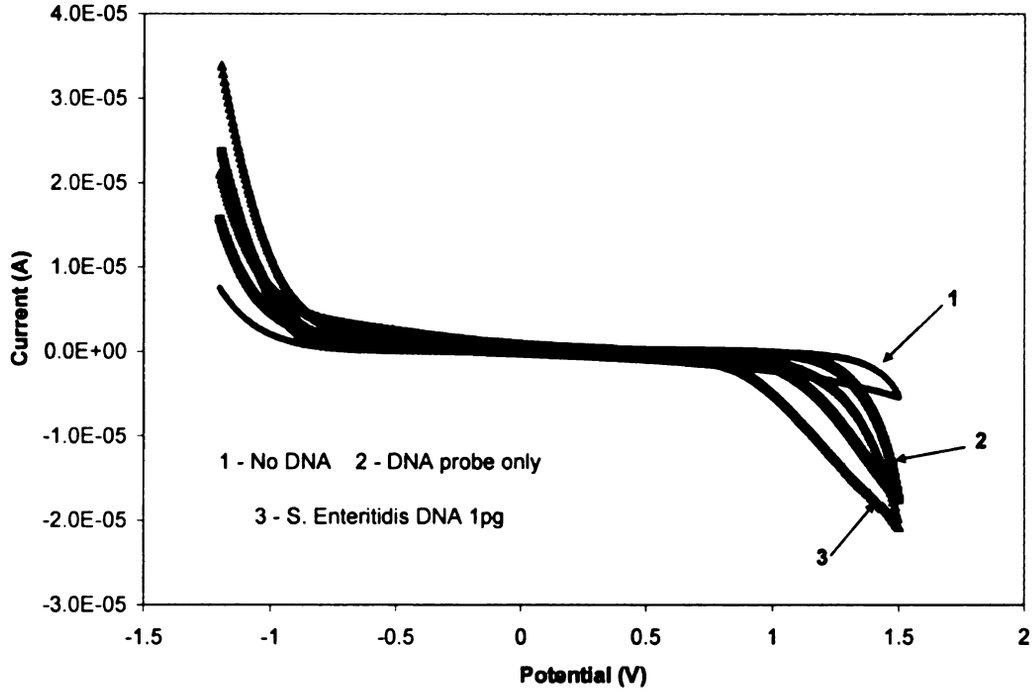

Figure C-21. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor chip for 1 pg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

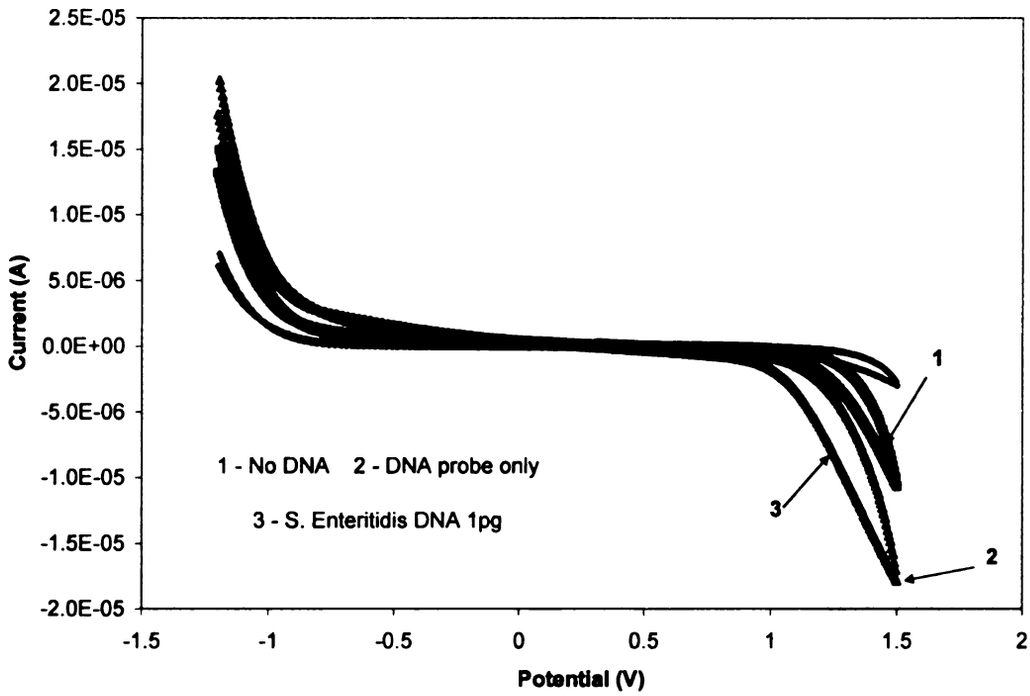

Figure C-22. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor chip for 1 pg of PCR-amplified *Salmonella* Enteritidis DNA (n = 3).

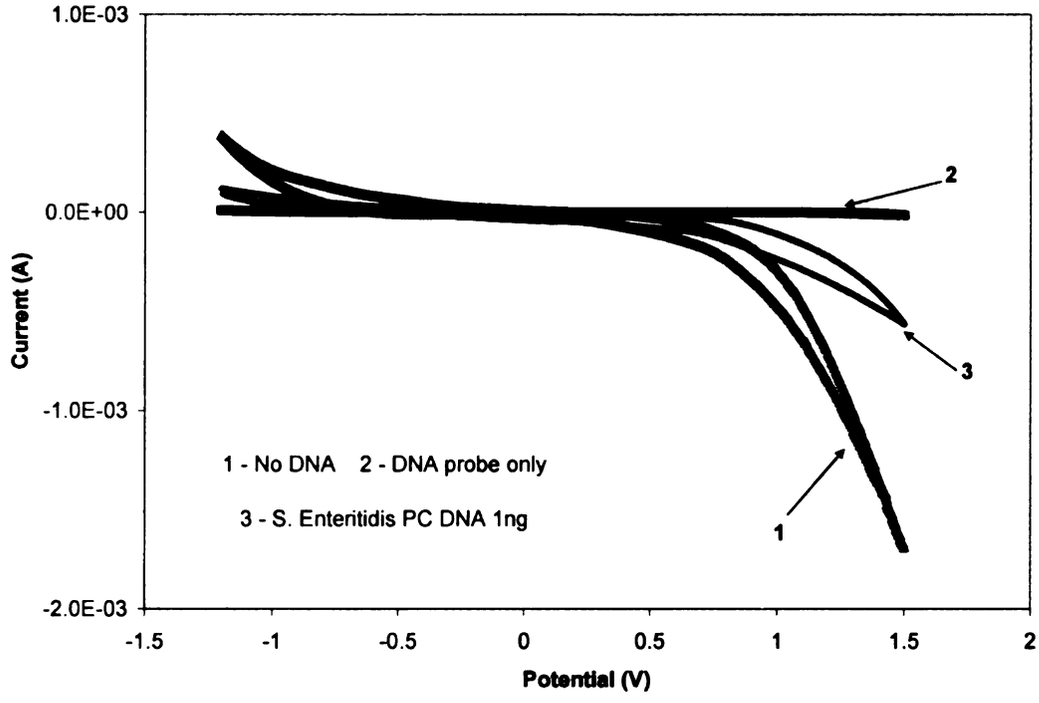

Figure C-23. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 ng of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

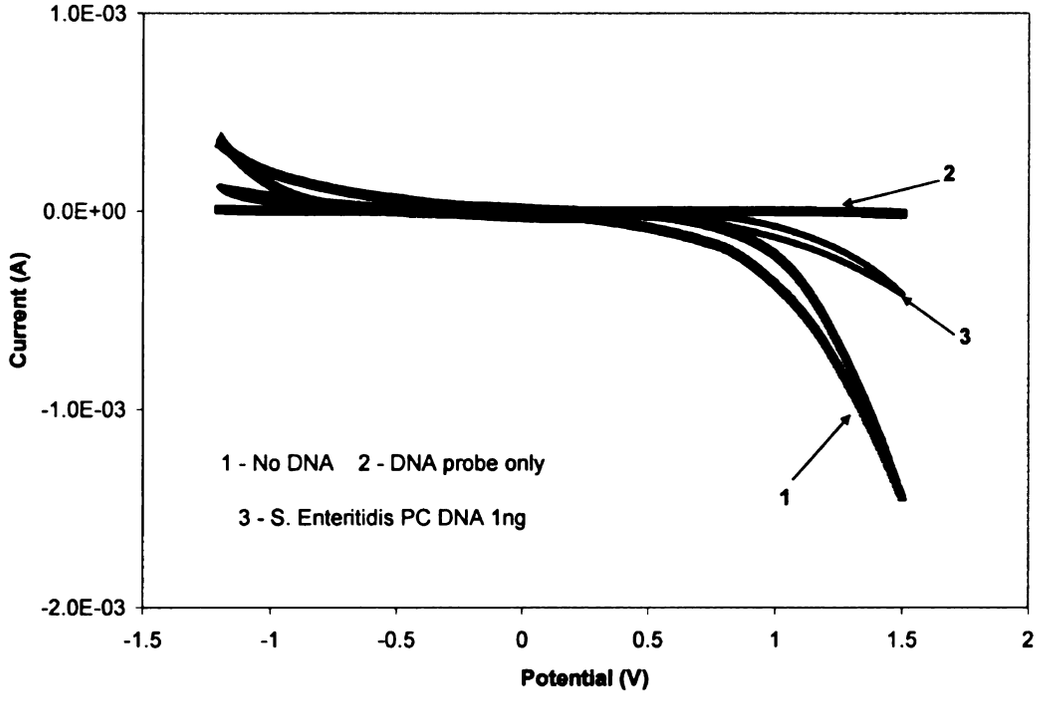

Figure C-24. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 ng of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

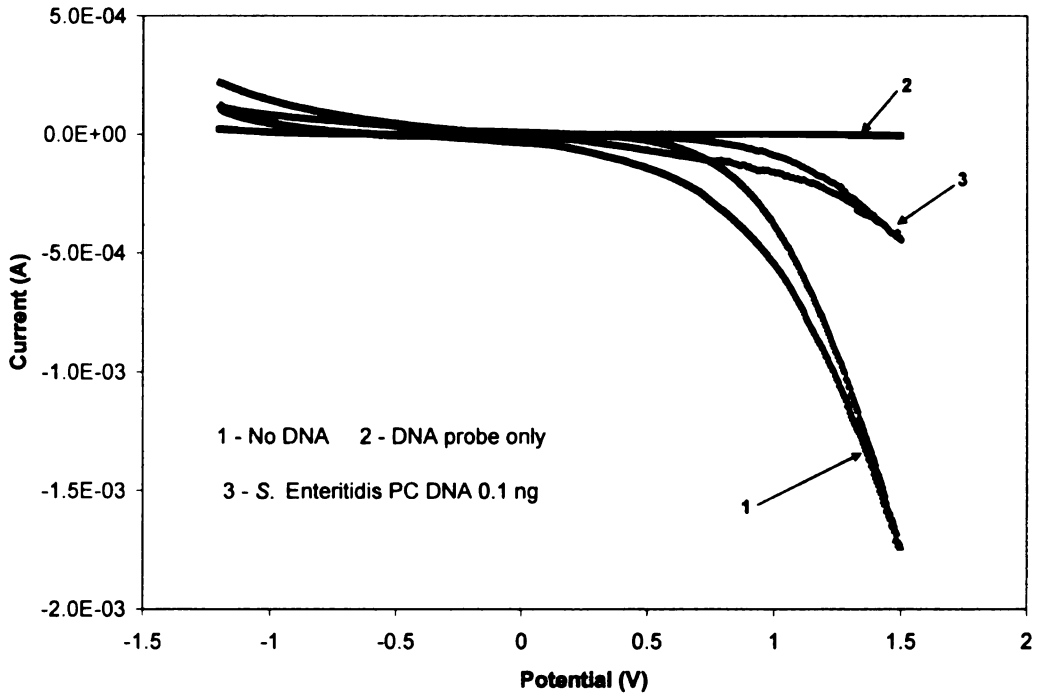

Figure C-25. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 0.1 ng of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

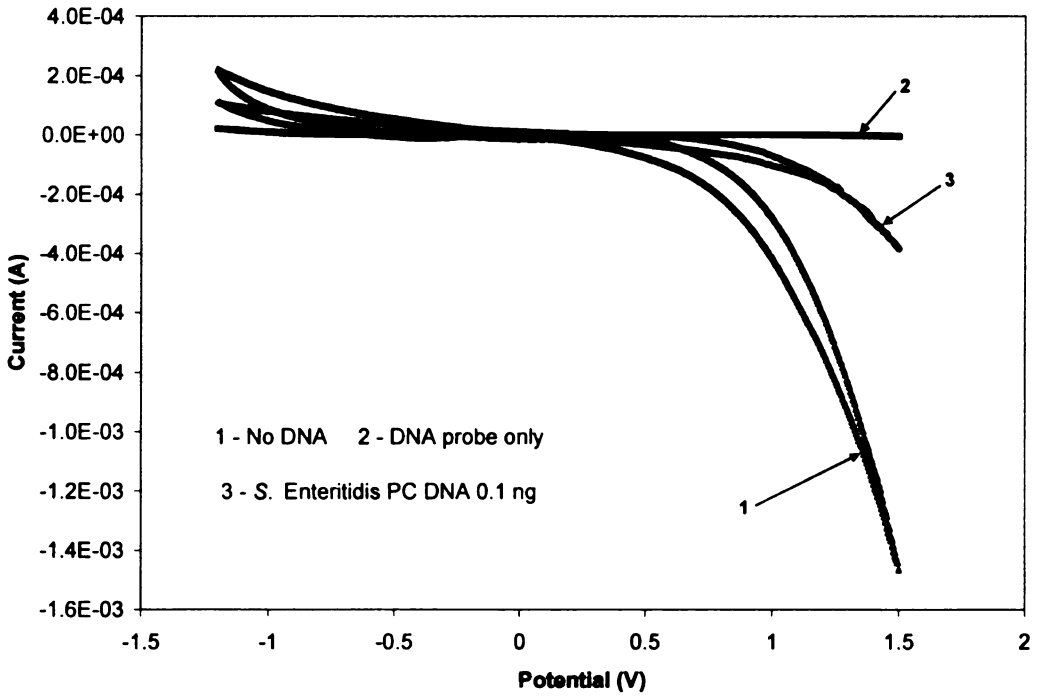

Figure C-26. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 0.1 ng of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

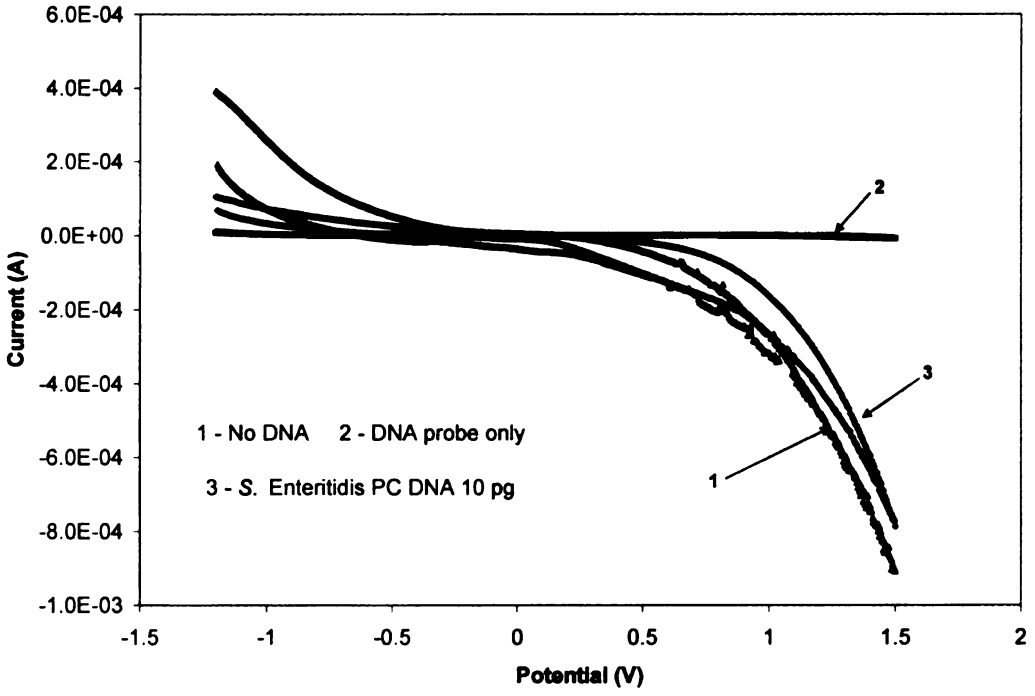

Figure C-27. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 10 pg of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

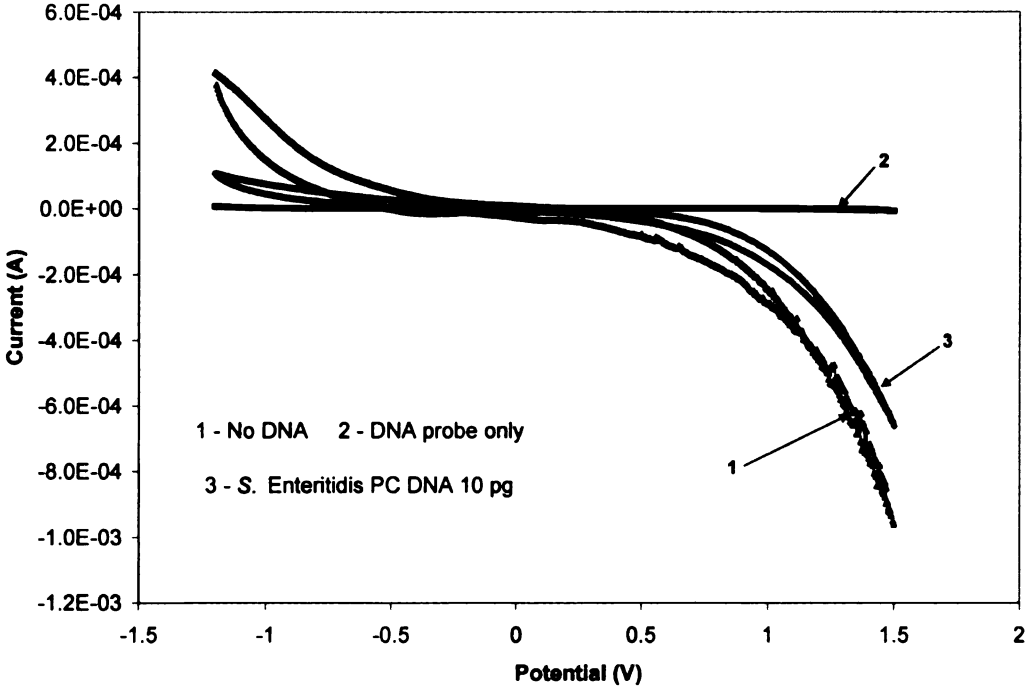

Figure C-28. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 10 pg of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

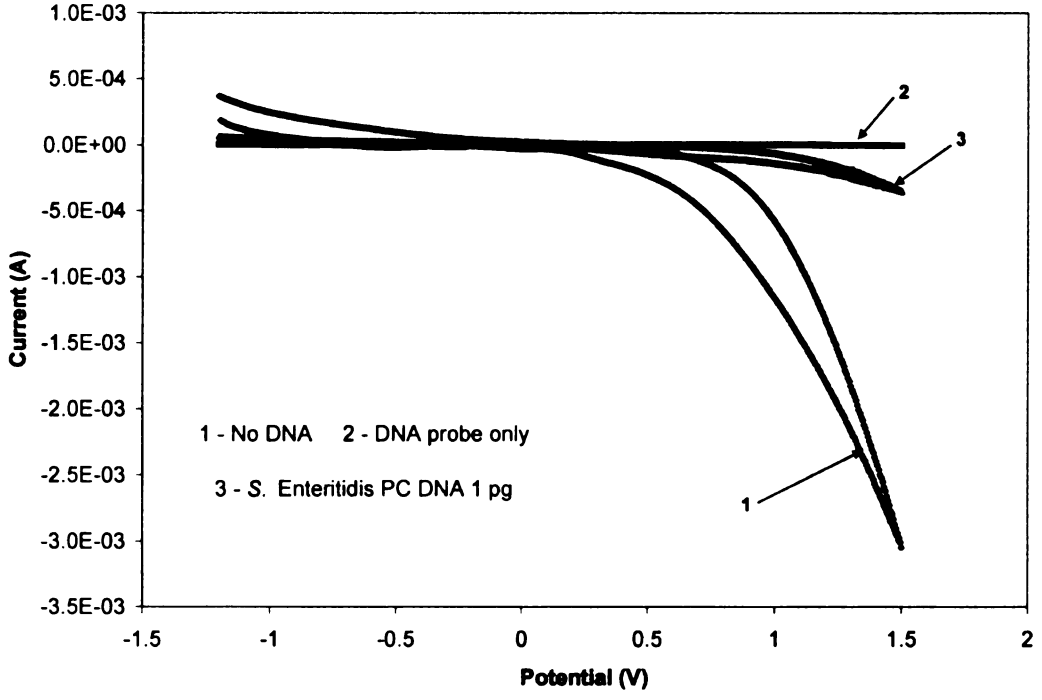

Figure C-29. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 pg of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

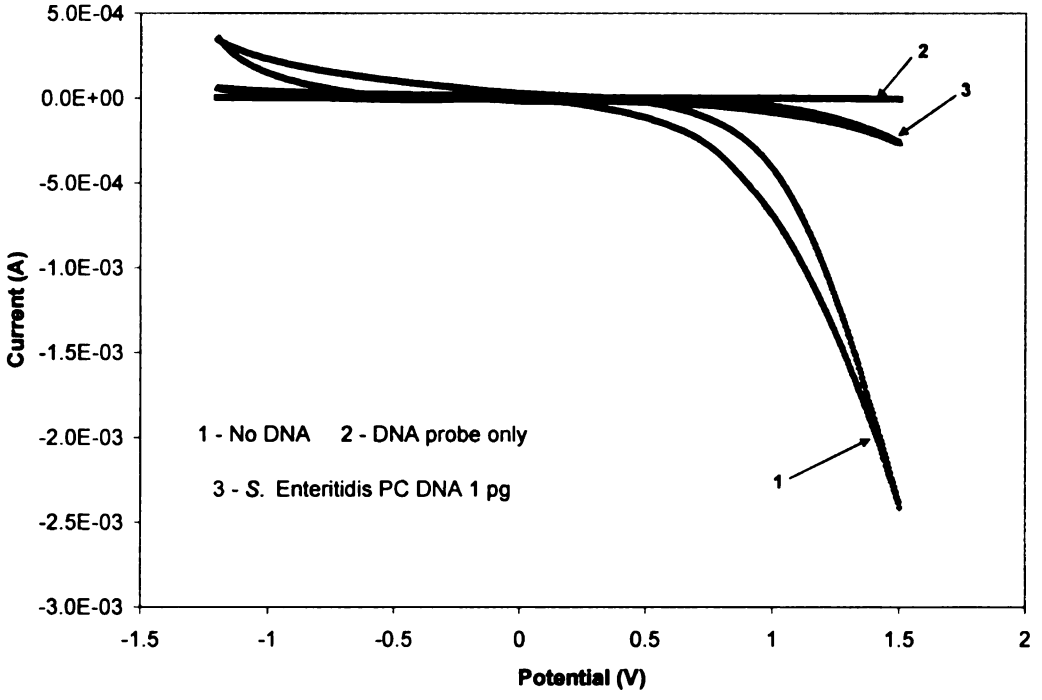

Figure C-30. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 pg of DNA extracted from pure culture of *Salmonella* Enteritidis (n = 3).

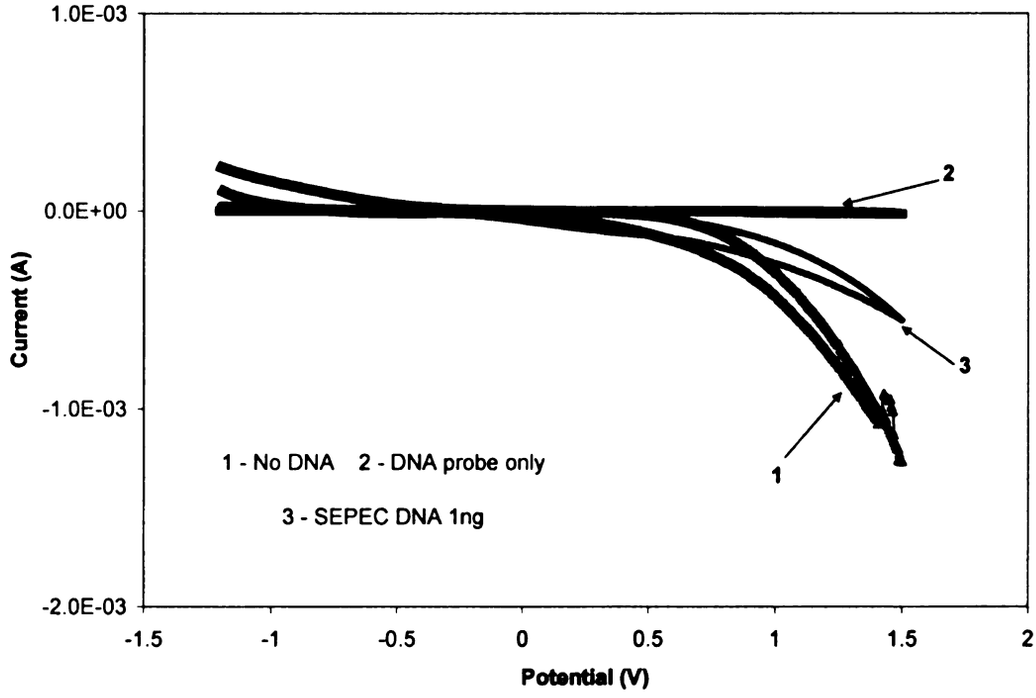

Figure C-31. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor for 1 ng mixture of *S.* Enteritidis and *E. coli* pure culture DNA (n = 3).

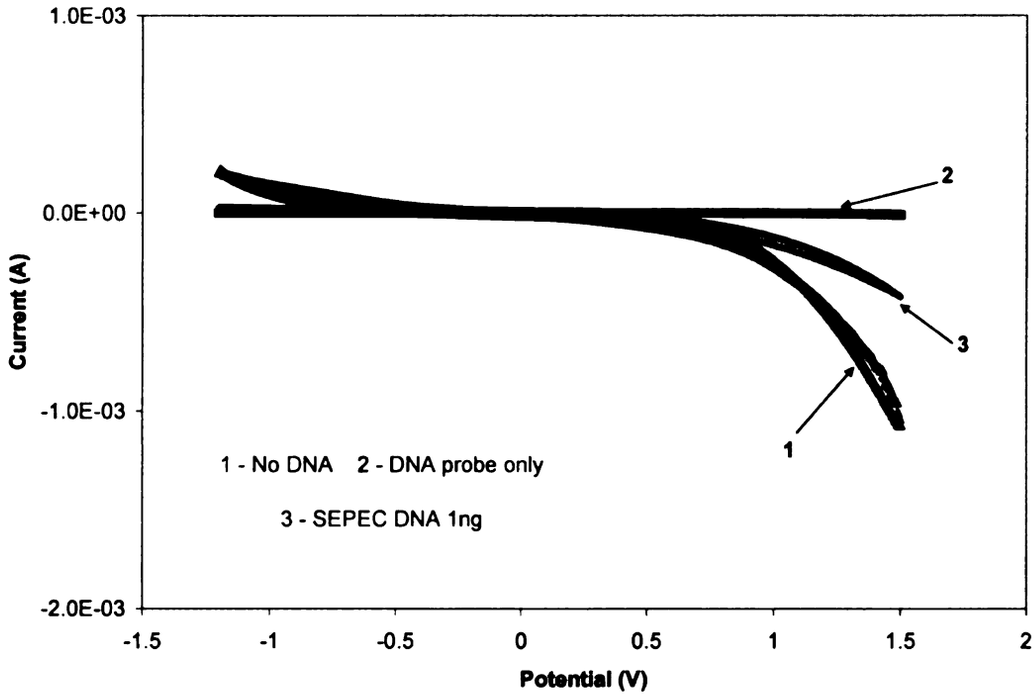

Figure C-32. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor for 1 ng mixture of *S.* Enteritidis and *E. coli* pure culture DNA (n = 3).

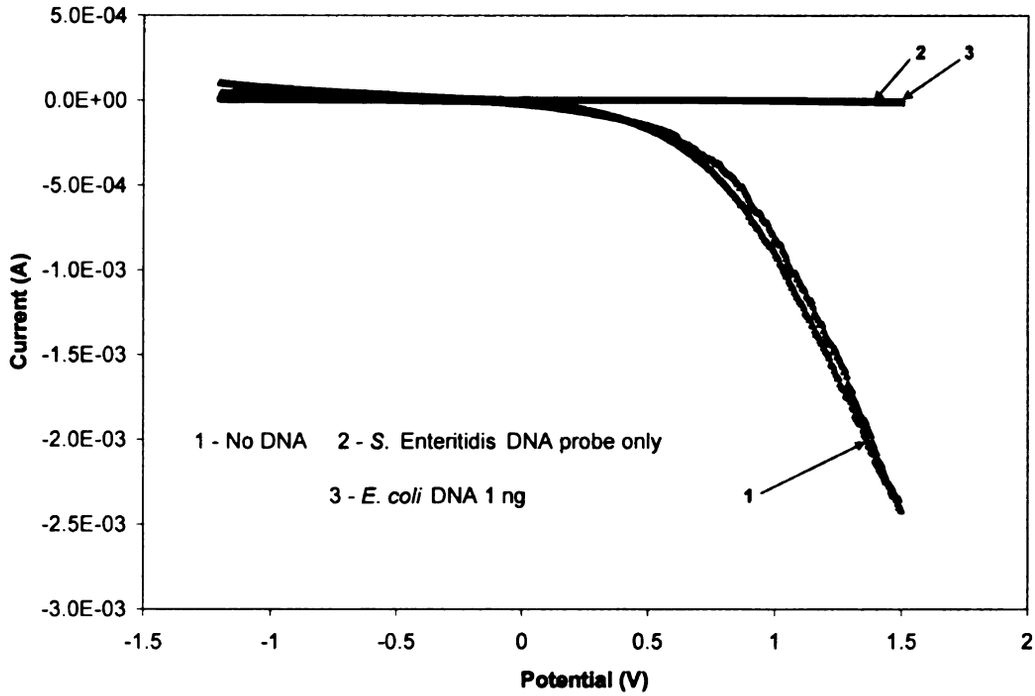

Figure C-33. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 ng of DNA extracted from pure culture of *E. coli* (n = 3).

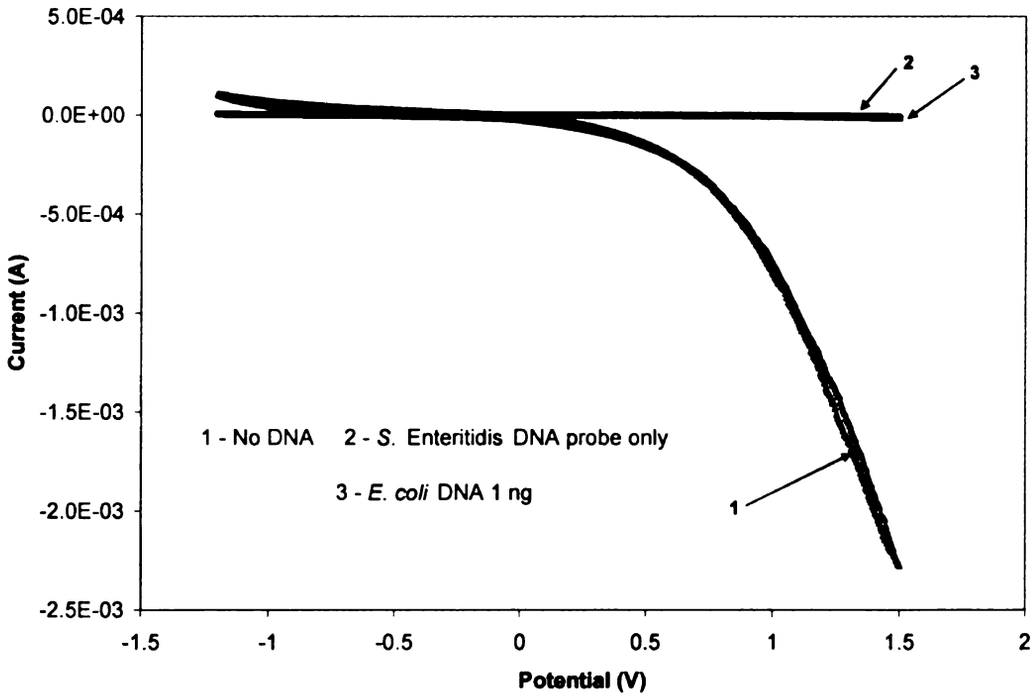

Figure C-34. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 ng of DNA extracted from pure culture of *E. coli* (n = 3).

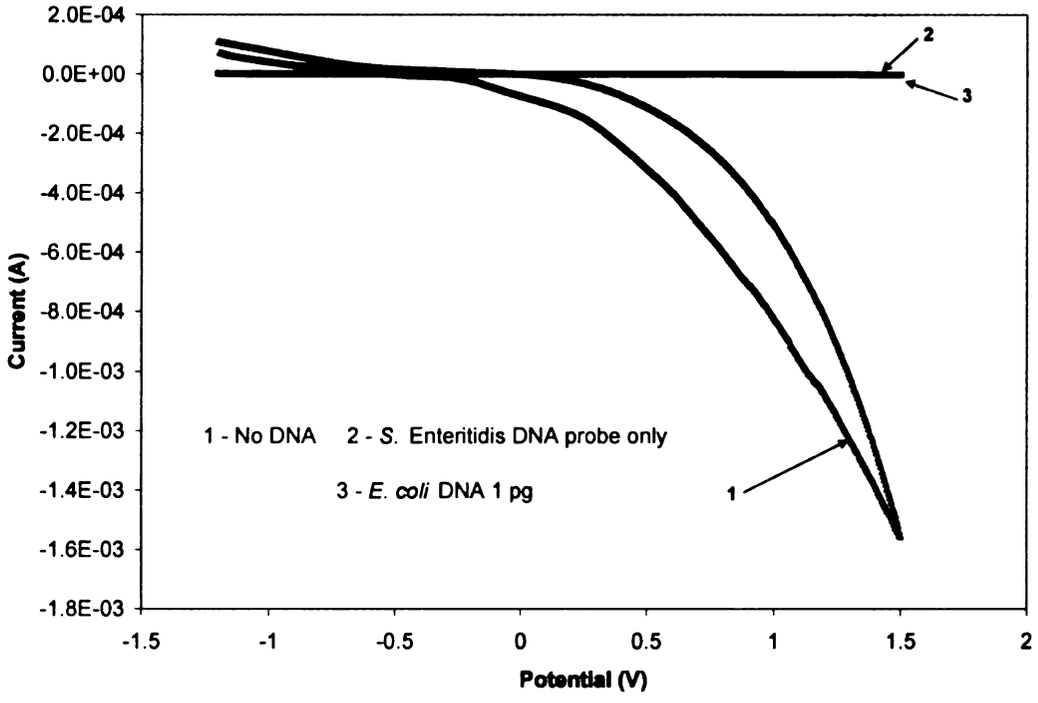

Figure C-35. Mean cyclic voltammograms (Cycle 1) obtained with NTS biosensor chip for 1 pg of DNA extracted from pure culture of *E. coli* (n = 3).

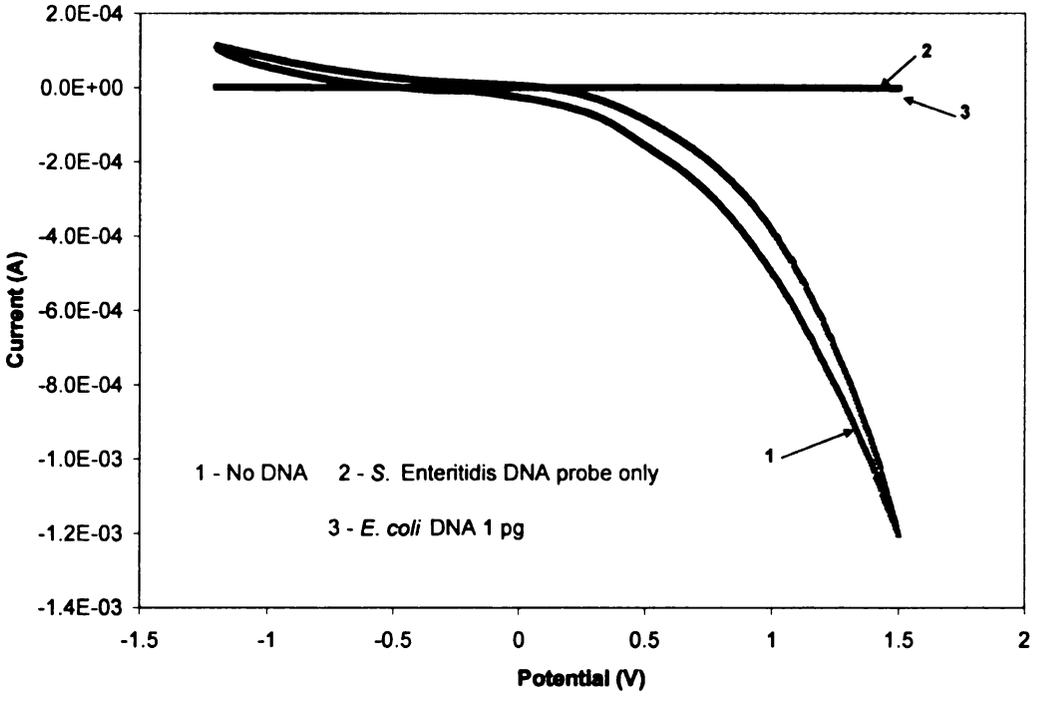

Figure C-36. Mean cyclic voltammograms (Cycle 2) obtained with NTS biosensor chip for 1 pg of DNA extracted from pure culture of *E. coli* (n = 3).

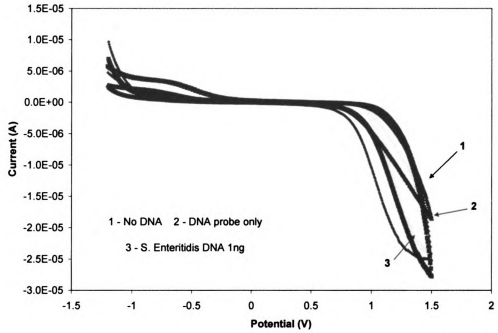

Figure C-37. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor chip for 1 ng of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

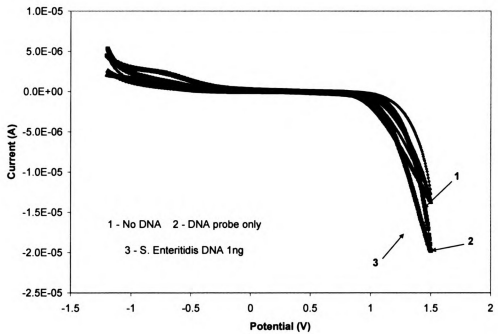

Figure C-38. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor chip for 1 ng of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

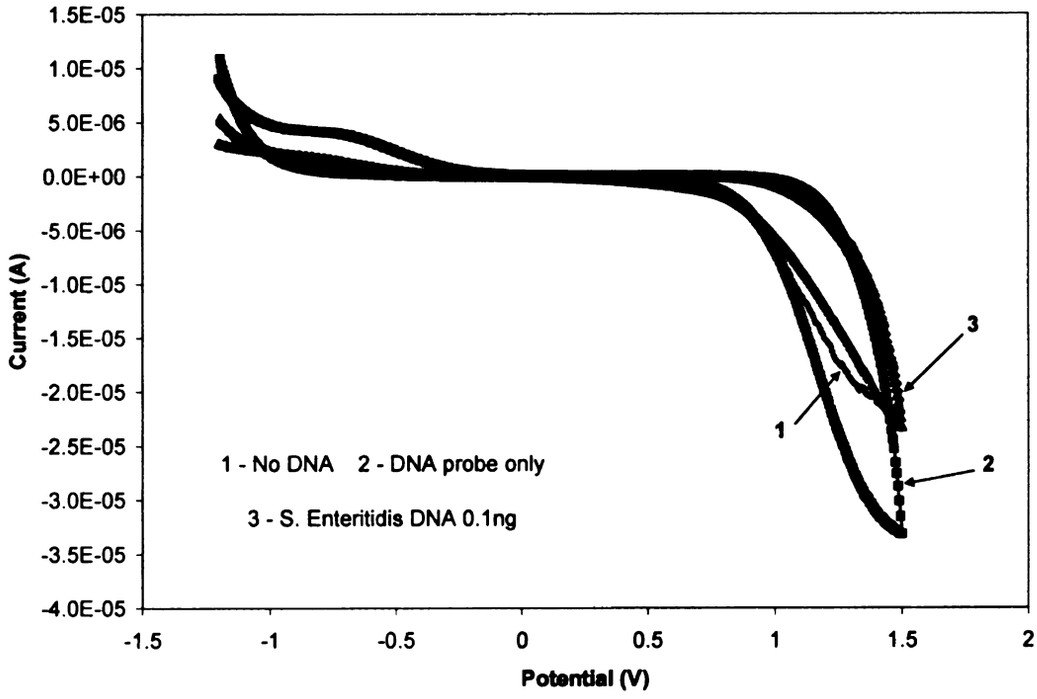

Figure C-39. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor for 0.1 ng of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

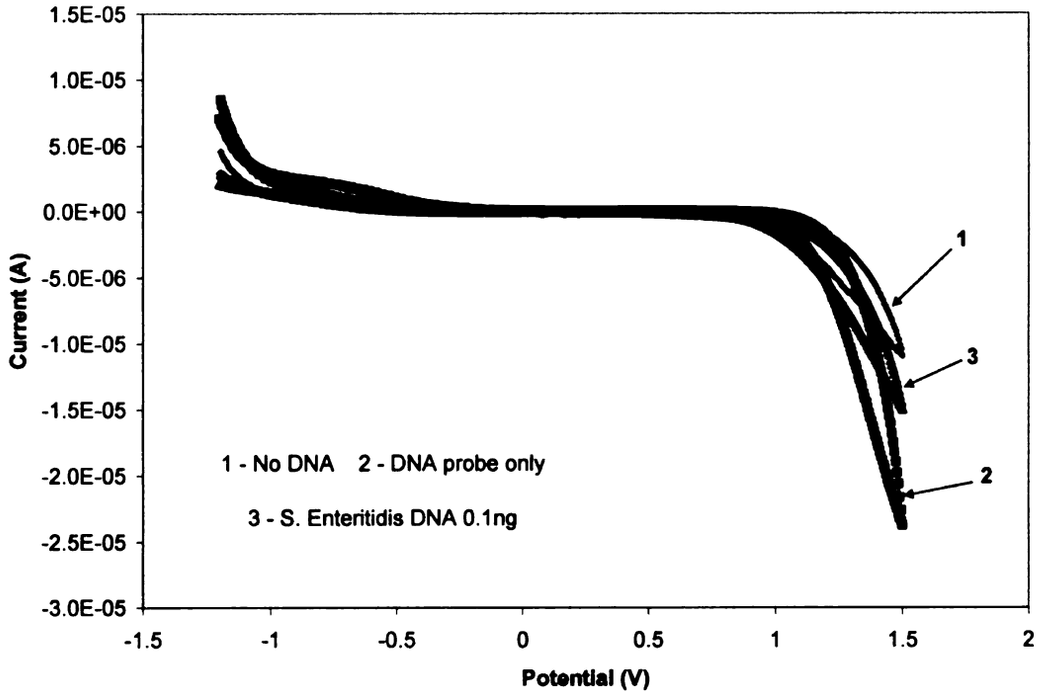

Figure C-40. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor for 0.1 ng of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

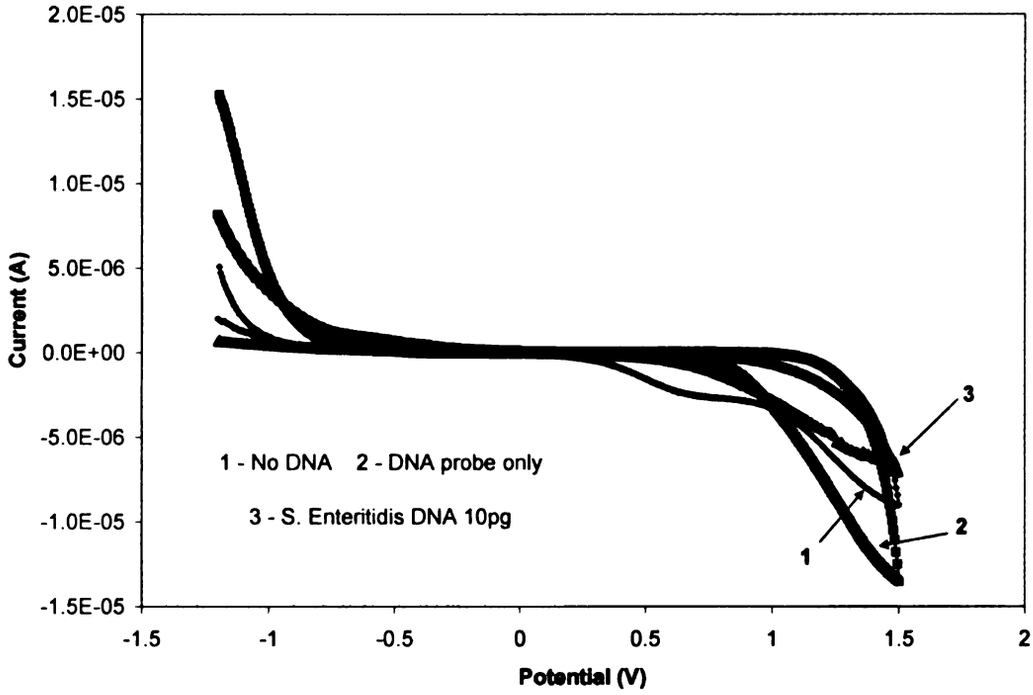

Figure C-41. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor for 10 pg of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

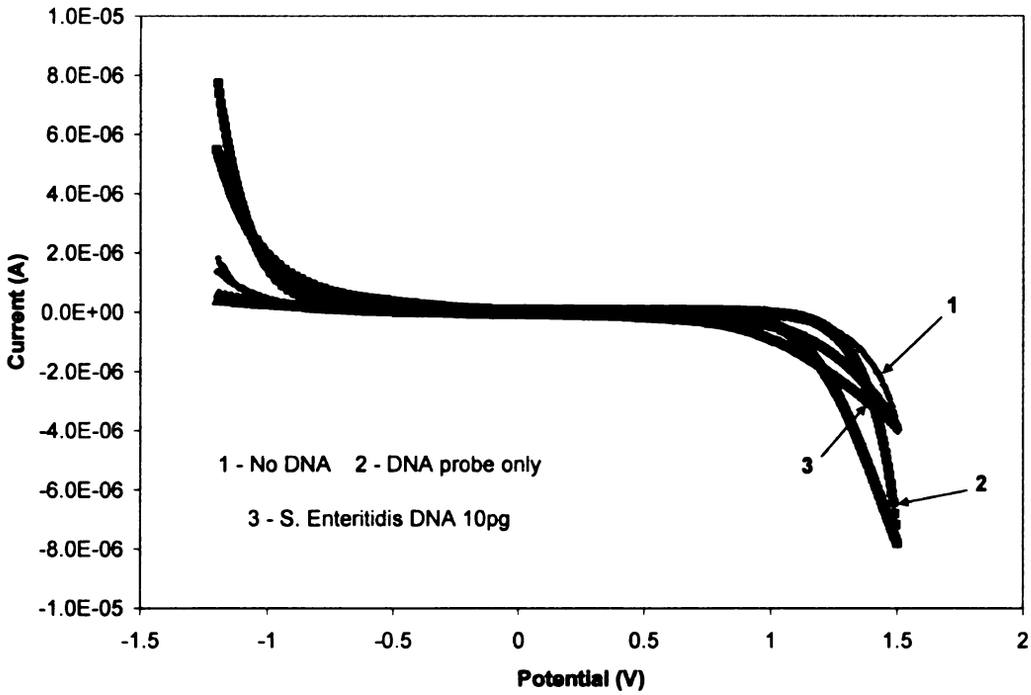

Figure C-42. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor for 10 pg of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

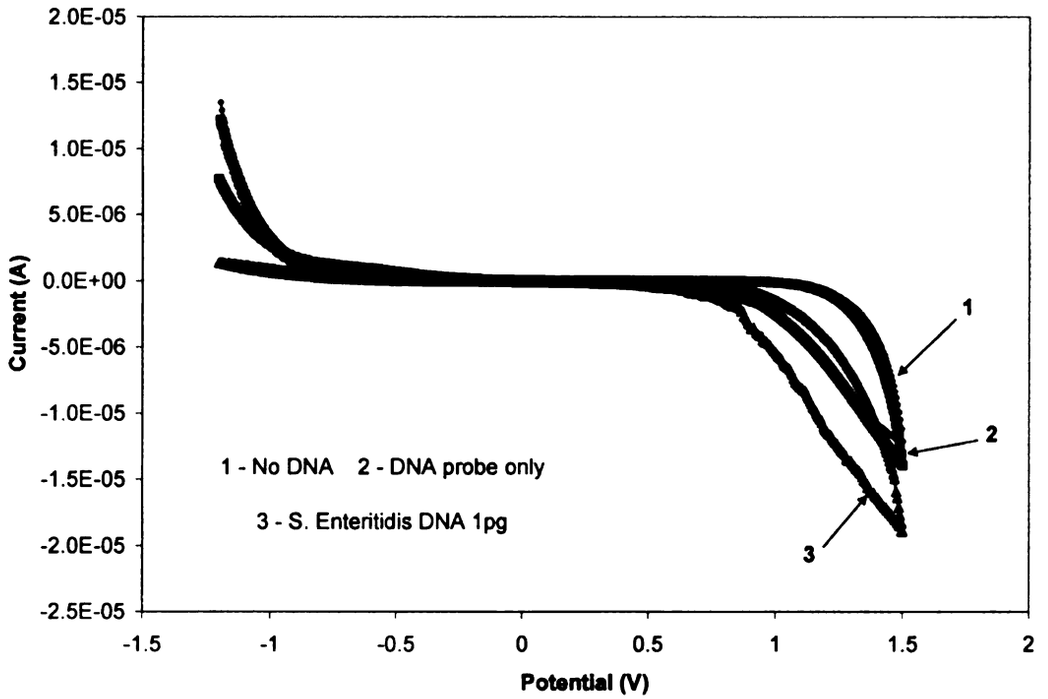

Figure C-43. Mean cyclic voltammograms (Cycle 1) obtained with Planar Si biosensor for 1 pg of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

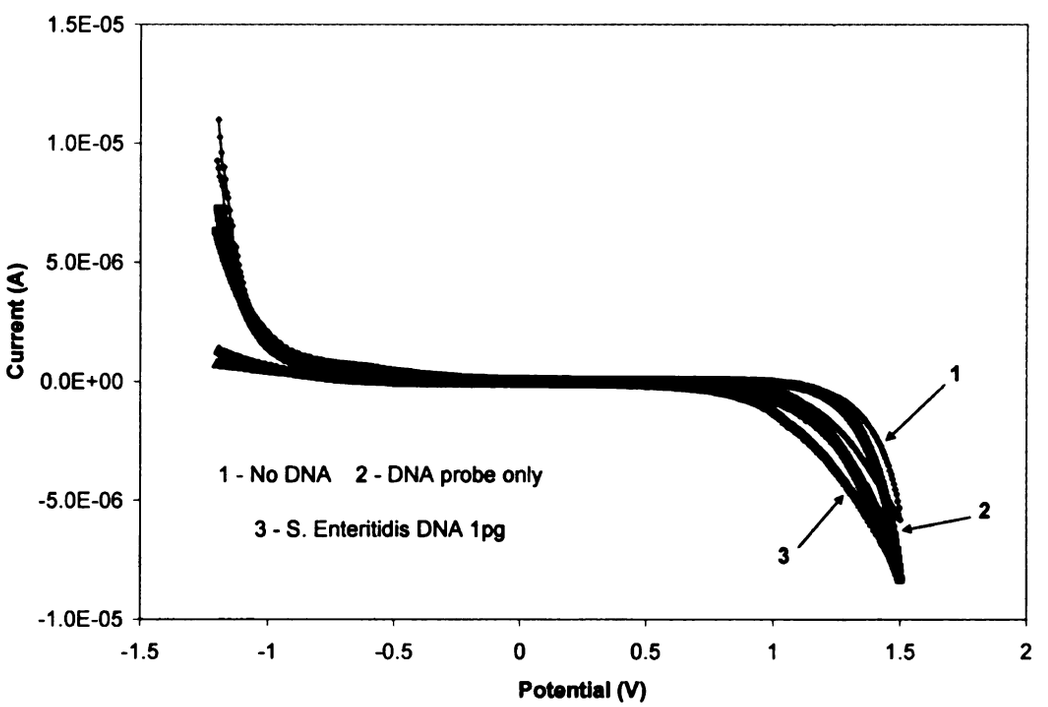

Figure C-44. Mean cyclic voltammograms (Cycle 2) obtained with Planar Si biosensor for 1 pg of DNA extracted from pure culture of *S.* Enteritidis (n = 3).

C.2 SAS Statistical Data Analysis Output

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=Ecoli DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.00008	3	After
A			
A	-0.00026	3	Before
B	-0.10267	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=Ecoli DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.00005	3	After
A			
A	-0.00005	3	Before
B	-0.07643	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SEPEC DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000456	3	Before
B	-0.043467	3	After
C	-0.052800	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000306	3	Before
B	-0.014467	3	After
C	-0.060767	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=0.1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000576	3	Before
A			
A	-0.005933	3	After
B	-0.023600	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=10fg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000076	3	After
A			
A	-0.000571	3	Before
B	-0.048400	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000237	3	Before
B	-0.012633	3	After
C	-0.029867	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1fg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000030	3	After
A			
A	-0.000461	3	Before
B	-0.015867	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000231	3	Before
B	-0.045767	3	After
C	-0.173333	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.00018	3	Before
A			
A	-0.00777	3	After
A			
A	-0.07717	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.0003390	3	Before
B	-0.0101133	3	After
C	-0.0667333	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.000008	3	Before
B	-0.026633	3	After
B			
B	-0.029500	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000028	3	Before
B	-0.030633	3	Blank
C	-0.075700	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.02588	3	After
A			
A	-0.09467	3	Before
A			
A	-0.10513	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.0000273	3	After
A			
A	-0.0000373	3	Before
A			
A	-0.0001669	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.00009380	3	Blank
A			
A	0.00009000	3	Before
A			
A	-.00002077	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.0001324	3	Before
A			
A	-0.0000145	3	After
A			
A	-0.0001973	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.00006933	3	After
A			
A	0.00003780	3	Blank
A			
A	0.00002203	3	Before

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.0000798	3	Before
A			
A	-0.0003072	3	Blank
A			
A	-0.0003300	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.0000812	3	Before
A			
A	-0.0002120	3	After
A			
A	-0.0002976	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	DNA_Hyb
	A	0.0000850	3	Blank
	A			
B	A	-0.0000881	3	Before
B				
B		-0.0004143	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=1 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.0001125	3	Blank
A			
A	-0.0001174	3	Before
A			
A	-0.0005023	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=Ecoli DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.00008	3	After
A			
A	-0.00024	3	Before
B	-0.09357	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=Ecoli DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.00004	3	Before
A			
A	-0.00005	3	After
B	-0.04983	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SEPEC DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000181	3	Before
B	-0.023733	3	After
C	-0.035600	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000249	3	Before
B	-0.013733	3	After
C	-0.056833	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=0.1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000210	3	Before
A			
A	-0.004770	3	After
B	-0.024133	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=10fg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000066	3	After
A			
A	-0.000349	3	Before
B	-0.049000	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000174	3	Before
A			
A	-0.009797	3	After
B	-0.025000	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1fg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000040	3	After
A			
A	-0.000192	3	Before
B	-0.014267	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000202	3	Before
B	-0.039667	3	After
C	-0.148000	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=PCR Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000153	3	Before
B	-0.006267	3	After
C	-0.051100	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.0003290	3	Before
B	-0.0096300	3	After
C	-0.0622667	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.000019	3	Before
B	-0.018300	3	After
B			
B	-0.024333	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.000030	3	Before
B	-0.028333	3	Blank
C	-0.045133	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=NTSi pathogen=SalEnt DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.01506	3	After
A			
A	-0.06880	3	Blank
A			
A	-0.09183	3	Before

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.00005627	3	Before
A			
A	0.00001102	3	After
A			
A	-.00000487	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.00005073	3	Before
A			
A	0.00001213	3	Blank
A			
A	-.00000320	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.0001743	3	Before
A			
A	-0.0000183	3	After
A			
A	-0.0000411	3	Blank

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=PCR Concentration=1pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	0.00007866	3	Before
A			
A	0.00003439	3	Blank
A			
A	0.00000429	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=0.1ng

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-0.0000555	3	Before
A			
A	-0.0001312	3	Blank
A			
A	-0.0003773	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=10pg

Means with the same letter are not significantly different.			
Tukey Grouping	Mean	N	DNA_Hyb
A	-.00001424	3	Before
A			
A	-.00005730	3	Blank
A			
A	-.00009533	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=1ng

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	DNA_Hyb
	A	0.00007303	3	Blank
	A			
B	A	-.00008284	3	Before
B				
B		-.00023633	3	After

The GLM Procedure: Tukey's Studentized Range (HSD) Test for DeltaQ

Tech_rep=2 platform=Si pathogen=SalEnt DNA_source=Pure Concentration=1pg

Means with the same letter are not significantly different.				
Tukey Grouping		Mean	N	DNA_Hyb
	A	0.00001679	3	Blank
	A			
B	A	-.00003363	3	Before
B				
B		-.00017043	3	After

The Mixed Procedure: Tech_rep=1 platform=NTSi pathogen=Ecoli

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	12	27.49	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	1	12	0.60	0.4526
DNA_Hyb*Concentratio	2	12	0.59	0.5719
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	-0.00009	0.01393	12	-0.01	0.9949
before - blank	0.08940	0.01393	12	6.42	<.0001
after - blank	0.08949	0.01393	12	6.42	<.0001
before - after|PCR	Non-est	.	.	.	.
before - blank|PCR	Non-est	.	.	.	.
after - blank|PCR	Non-est	.	.	.	.
before - after|pure	Non-est	.	.	.	.
before - blank|pure	Non-est	.	.	.	.
after - blank|pure	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=1 platform=NTSi pathogen=SEPEC

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	6	498.80	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	0	.	.	.
DNA_Hyb*Concentratio	0	.	.	.
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.04301	0.001768	6	24.33	<.0001
before - blank	0.05234	0.001768	6	29.61	<.0001
after - blank	0.009333	0.001768	6	5.28	0.0019
before - after|PCR	Non-est	.	.	.	.
before - blank|PCR	Non-est	.	.	.	.
after - blank|PCR	Non-est	.	.	.	.
before - after|pure	Non-est	.	.	.	.
before - blank|pure	Non-est	.	.	.	.
after - blank|pure	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=1 platform=NTSi pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	48	17.10	<.0001
DNA_source	1	48	0.16	0.6882
DNA_Hyb*DNA_source	2	48	3.25	0.0475
Concentration	3	48	4.78	0.0054
DNA_Hyb*Concentratio	6	48	2.29	0.0502
DNA_sourc*Concentrat	3	48	3.99	0.0130
DNA_Hy*DNA_so*Concen	6	48	2.74	0.0225

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.01546	0.01060	48	1.46	0.1514
before - blank	0.05973	0.01060	48	5.63	<.0001
after - blank	0.04427	0.01060	48	4.18	0.0001
before - after|PCR	0.01992	0.01499	48	1.33	0.1903
before - blank|PCR	0.08504	0.01499	48	5.67	<.0001
after - blank|PCR	0.06512	0.01499	48	4.34	<.0001
before - after|pure	0.01099	0.01499	48	0.73	0.4670
before - blank|pure	0.03441	0.01499	48	2.29	0.0262
after - blank|pure	0.02342	0.01499	48	1.56	0.1249

The Mixed Procedure: Tech_rep=1 platform=Si pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	48	4.66	0.0141
DNA_source	1	48	15.13	0.0003
DNA_Hyb*DNA_source	2	48	3.26	0.0469
Concentration	3	48	0.29	0.8335
DNA_Hyb*Concentratio	6	48	0.65	0.6884
DNA_sourc*Concentrat	3	48	0.90	0.4473
DNA_Hy*DNA_so*Concen	6	48	1.77	0.1250

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.000182	0.000060	48	3.04	0.0039
before - blank	0.000108	0.000060	48	1.81	0.0768
after - blank	-0.00007	0.000060	48	-1.23	0.2259
before - after|PCR	0.000050	0.000085	48	0.59	0.5565
before - blank|PCR	0.000110	0.000085	48	1.30	0.1998
after - blank|PCR	0.000060	0.000085	48	0.71	0.4825
before - after|pure	0.000313	0.000085	48	3.70	0.0006
before - blank|pure	0.000106	0.000085	48	1.26	0.2146
after - blank|pure	-0.00021	0.000085	48	-2.44	0.0183

The Mixed Procedure: Tech_rep=2 platform=NTSi pathogen=Ecoli

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	12	25.30	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	1	12	2.38	0.1485
DNA_Hyb*Concentratio	2	12	2.35	0.1379
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	-0.00008	0.01162	12	-0.01	0.9948
before - blank	0.07156	0.01162	12	6.16	<.0001
after - blank	0.07164	0.01162	12	6.16	<.0001
before - after|PCR	Non-est	.	.	.	.
before - blank|PCR	Non-est	.	.	.	.
after - blank|PCR	Non-est	.	.	.	.
before - after|pure	Non-est	.	.	.	.
before - blank|pure	Non-est	.	.	.	.
after - blank|pure	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=2 platform=NTSi pathogen=SEPEC

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	6	107.60	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	0	.	.	.
DNA_Hyb*Concentratio	0	.	.	.
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.02355	0.002458	6	9.58	<.0001
before - blank	0.03542	0.002458	6	14.41	<.0001
after - blank	0.01187	0.002458	6	4.83	0.0029
before - after|PCR	Non-est	.	.	.	.
before - blank|PCR	Non-est	.	.	.	.
after - blank|PCR	Non-est	.	.	.	.
before - after|pure	Non-est	.	.	.	.
before - blank|pure	Non-est	.	.	.	.
after - blank|pure	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=2 platform=NTSi pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	48	13.46	<.0001
DNA_source	1	48	0.02	0.8972
DNA_Hyb*DNA_source	2	48	3.06	0.0561
Concentration	3	48	3.23	0.0304
DNA_Hyb*Concentratio	6	48	2.29	0.0502
DNA_sourc*Concentrat	3	48	4.10	0.0114
DNA_Hy*DNA_so*Concen	6	48	2.21	0.0581

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.008163	0.009580	48	0.85	0.3984
before - blank	0.04655	0.009580	48	4.86	<.0001
after - blank	0.03839	0.009580	48	4.01	0.0002
before - after|PCR	0.01717	0.01355	48	1.27	0.2111
before - blank|PCR	0.07004	0.01355	48	5.17	<.0001
after - blank|PCR	0.05287	0.01355	48	3.90	0.0003
before - after|pure	-0.00085	0.01355	48	-0.06	0.9505
before - blank|pure	0.02306	0.01355	48	1.70	0.0952
after - blank|pure	0.02390	0.01355	48	1.76	0.0840

The Mixed Procedure: Tech_rep=2 platform=Si pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	48	7.18	0.0019
DNA_source	1	48	18.24	<.0001
DNA_Hyb*DNA_source	2	48	3.58	0.0355
Concentration	3	48	1.28	0.2924
DNA_Hyb*Concentratio	6	48	0.41	0.8700
DNA_soure*Concentrat	3	48	0.95	0.4227
DNA_Hy*DNA_so*Concen	6	48	1.09	0.3800

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after	0.000132	0.000036	48	3.65	0.0006
before - blank	0.000034	0.000036	48	0.94	0.3539
after - blank	-0.00010	0.000036	48	-2.71	0.0093
before - after|PCR	0.000092	0.000051	48	1.78	0.0809
before - blank|PCR	0.000090	0.000051	48	1.75	0.0865
after - blank|PCR	-1.69E-6	0.000051	48	-0.03	0.9739
before - after|pure	0.000173	0.000051	48	3.38	0.0015
before - blank|pure	-0.00002	0.000051	48	-0.43	0.6717
after - blank|pure	-0.00020	0.000051	48	-3.80	0.0004

The Mixed Procedure: Tech_rep=1 pathogen=Ecoli

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	12	27.49	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	1	12	0.60	0.4526
DNA_Hyb*Concentratio	2	12	0.59	0.5719
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.
platform	0	.	.	.
DNA_Hyb*platform	0	.	.	.
DNA_source*platform	0	.	.	.
DNA_Hy*DNA_so*platfo	0	.	.	.
Concentrati*platform	0	.	.	.
DNA_Hy*Concen*platfo	0	.	.	.
DNA_so*Concen*platfo	0	.	.	.
DNA_*DNA_*Conc*platf	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	Non-est	.	.	.	.
before - blank|NTSI	Non-est	.	.	.	.
after - blank|NTSI	Non-est	.	.	.	.
before - after|SI	Non-est	.	.	.	.
before - blank|SI	Non-est	.	.	.	.
after - blank|SI	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=1 pathogen=SEPEC

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	6	498.80	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	0	.	.	.
DNA_Hyb*Concentratio	0	.	.	.
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.
platform	0	.	.	.
DNA_Hyb*platform	0	.	.	.
DNA_source*platform	0	.	.	.
DNA_Hy*DNA_so*platfo	0	.	.	.
Concentrati*platform	0	.	.	.
DNA_Hy*Concen*platfo	0	.	.	.
DNA_so*Concen*platfo	0	.	.	.
DNA_*DNA_*Conc*platf	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	Non-est	.	.	.	.
before - blank|NTSI	Non-est	.	.	.	.
after - blank|NTSI	Non-est	.	.	.	.
before - after|SI	Non-est	.	.	.	.
before - blank|SI	Non-est	.	.	.	.
after - blank|SI	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=1 pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	96	17.13	<.0001
DNA_source	1	96	0.18	0.6713
DNA_Hyb*DNA_source	2	96	3.26	0.0426
Concentration	3	96	4.78	0.0038
DNA_Hyb*Concentratio	6	96	2.29	0.0412
DNA_sourc*Concentrat	3	96	4.00	0.0099
DNA_Hy*DNA_so*Concen	6	96	2.76	0.0162
platform	1	96	72.58	<.0001
DNA_Hyb*platform	2	96	17.06	<.0001
DNA_source*platform	1	96	0.15	0.7035
DNA_Hy*DNA_so*platfo	2	96	3.24	0.0437
Concentrati*platform	3	96	4.78	0.0038
DNA_Hy*Concen*platfo	6	96	2.29	0.0412
DNA_so*Concen*platfo	3	96	3.97	0.0103
DNA_*DNA_*Conc*platf	6	96	2.73	0.0172

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	0.01546	0.007498	96	2.06	0.0420
before - blank|NTSI	0.05973	0.007498	96	7.97	<.0001
after - blank|NTSI	0.04427	0.007498	96	5.90	<.0001
before - after|SI	0.000182	0.007498	96	0.02	0.9807
before - blank|SI	0.000108	0.007498	96	0.01	0.9885
after - blank|SI	-0.00007	0.007498	96	-0.01	0.9922

The Mixed Procedure: Tech_rep=2 pathogen=Ecoli

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	12	25.30	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	1	12	2.38	0.1485
DNA_Hyb*Concentratio	2	12	2.35	0.1379
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.
platform	0	.	.	.
DNA_Hyb*platform	0	.	.	.
DNA_source*platform	0	.	.	.
DNA_Hy*DNA_so*platfo	0	.	.	.
Concentrati*platform	0	.	.	.
DNA_Hy*Concen*platfo	0	.	.	.
DNA_so*Concen*platfo	0	.	.	.
DNA_*DNA_*Conc*platf	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	Non-est	.	.	.	.
before - blank|NTSI	Non-est	.	.	.	.
after - blank|NTSI	Non-est	.	.	.	.
before - after|SI	Non-est	.	.	.	.
before - blank|SI	Non-est	.	.	.	.
after - blank|SI	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=2 pathogen=SEPEC

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	6	107.60	<.0001
DNA_source	0	.	.	.
DNA_Hyb*DNA_source	0	.	.	.
Concentration	0	.	.	.
DNA_Hyb*Concentratio	0	.	.	.
DNA_sourc*Concentrat	0	.	.	.
DNA_Hy*DNA_so*Concen	0	.	.	.
platform	0	.	.	.
DNA_Hyb*platform	0	.	.	.
DNA_source*platform	0	.	.	.
DNA_Hy*DNA_so*platfo	0	.	.	.
Concentrati*platform	0	.	.	.
DNA_Hy*Concen*platfo	0	.	.	.
DNA_so*Concen*platfo	0	.	.	.
DNA_*DNA_*Conc*platf	0	.	.	.

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	Non-est	.	.	.	.
before - blank|NTSI	Non-est	.	.	.	.
after - blank|NTSI	Non-est	.	.	.	.
before - after|SI	Non-est	.	.	.	.
before - blank|SI	Non-est	.	.	.	.
after - blank|SI	Non-est	.	.	.	.

The Mixed Procedure: Tech_rep=2 pathogen=SalEnt

Type 3 Tests of Fixed Effects				
Effect	Num DF	Den DF	F Value	Pr > F
DNA_Hyb	2	96	13.46	<.0001
DNA_source	1	96	0.02	0.8842
DNA_Hyb*DNA_source	2	96	3.08	0.0506
Concentration	3	96	3.23	0.0259
DNA_Hyb*Concentratio	6	96	2.29	0.0412
DNA_sourc*Concentrat	3	96	4.09	0.0088
DNA_Hy*DNA_so*Concen	6	96	2.22	0.0478
platform	1	96	57.82	<.0001
DNA_Hyb*platform	2	96	13.47	<.0001
DNA_source*platform	1	96	0.01	0.9097
DNA_Hy*DNA_so*platfo	2	96	3.04	0.0523
Concentrati*platform	3	96	3.24	0.0256
DNA_Hy*Concen*platfo	6	96	2.29	0.0413
DNA_so*Concen*platfo	3	96	4.10	0.0088
DNA_*DNA_*Conc*platf	6	96	2.20	0.0492

Estimates					
Label	Estimate	Standard Error	DF	t Value	Pr > |t|
before - after|NTSI	0.008163	0.006774	96	1.21	0.2312
before - blank|NTSI	0.04655	0.006774	96	6.87	<.0001
after - blank|NTSI	0.03839	0.006774	96	5.67	<.0001
before - after|SI	0.000132	0.006774	96	0.02	0.9844
before - blank|SI	0.000034	0.006774	96	0.01	0.9960
after - blank|SI	-0.00010	0.006774	96	-0.01	0.9884

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